Herpes Simplex Virus Type 1 (HSV-1) a Versatile Tool: Live Events in the HSV-1 Life Cycle and Applications on Gene and Protein Delivery

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Summary

The study of viruses has brought advances in many biological areas. Understanding the interactions of viruses with their environment, i.e., the host cell, allows the improvement of vaccines against viral infections and also the use and development of viruses as vectors of therapeutic genes and proteins. The present work focused on the use of HSV-1 as a carrier that could encode and/or deliver functional proteins. In order to evaluate the feasibility of using a recombinant herpes simplex virus type 1 (rHSV-1) virion to deliver foreign proteins, a rHSV-1 that simultaneously encodes selected structural proteins from all three virion compartments -capsid, tegument, and envelope- fused with autofluorescent proteins was constructed. Specifically, the triple-fluorescent recombinant, rHSV35R48C22Y, encoded the capsid VP26 protein fused to monomeric red fluorescent protein (mRFP), the tegument VP16 protein fused to enhanced cyan fluorescent protein (ECFP), and the envelope glycoprotein H fused to enhanced yellow fluorescent protein (EYFP). rHSV35R48C22Y was demonstrated to be fully replication-competent, although with delayed kinetics, incorporated the fusion proteins into all three virion compartments, and was comparable to wild-type HSV-1 at the ultrastructural level. Live imaging of cells infected with rHSV35R48C22Y also provided novel insights into the dynamics of compartmentalization and interaction among viral proteins.

As an extension of this study, and aiming to monitor the process of HSV-1 DNA delivery into the nucleus, a visualization system based on HSV-1 amplicon vectors and lac operator (lacO)-Lac repressor (LacR) interactions was developed. Briefly, HSV-1 amplicon vectors containing lacO sequences consisting of lacR binding sites, were packaged into recombinant HSV-1 virions encoding the VP26 capsid protein fused with mRFP (HSV-1 amplicon-35R/lacO), and used to infect cells that stably express nuclear LacR fused to the green fluorescent protein (MRC5/LacR-GPF). Live visualization of infected cells allowed tracing of HSV-1 amplicon-35R/lacO particles in the cytoplasm, however, the exact moment of HSV-1 amplicon vector DNA entry into the nucleus was not observed. Nevertheless, bright green foci corresponding to binding of the LacR-GFP to the lacR binding sites present on the HSV-1 amplicon genome were observed in fixed infected cell nuclei, and were demonstrated to develop into replication compartments.

A possible application for the delivery of foreign proteins by the virus particle was also exploited in this work. The first approach related to the development of a HSV-1 vector that delivers the therapeutic transgene DNA into the target cell, and simultaneously carries enzymatic functions fused to tegument- or capsid proteins. Specifically, a recombinant HSV-1 encoding the tegument VP16 protein fused to the AAV2 Rep protein was constructed, and demonstrated to express the VP16-Rep fusion protein. Replication assays showed that the Rep delivered by the VP16-Rep fusion protein in the virion tegument was able to support replication of a transgene cassette flanked by the AAV2 ITRs.

A second approach on combining genetic elements from HSV-1 and AAV2 was to construct a rHSV/AAV hybrid vector that mediates the *in situ* production of rAAV2 vectors in rHSV-1 vector-infected cells. The construction of this vector was aimed at increasing the distribution of therapeutic transgenes in solid brain tumors.

Zusammenfassung

Die virologische Forschung bringt Fortschritte in den verschiedensten biologischen Bereichen. Das Verständnis der Interaktion des Virus mit seiner Umgebung ermöglicht zum Beispiel die Verbesserung von Impfstoffen gegen virale Infektionen sowie die Verwendung und Entwicklung von Viren als Vektoren von therapeutischen Genen und Proteinen. Die vorliegende Arbeit behandelt im Schwerpunkt die Verwendung des Herpes simplex Virus Typ 1 (HSV-1) als einen Vektor, der funktionale Proteine codieren und/oder in eine Zelle einbringen kann. Um die Realisierbarkeit der Nutzung eines rekombinanten (r)HSV-1 zur Übertragung von fremden Proteinen untersuchen zu können, wurde ein rHSV-1 konstruiert, der simultan spezielle strukturelle Proteine der drei verschiedenen Viruseinheiten – Kapsid, Tegument und Mantel – gebunden an selbstfluoreszierende Proteine enthält. Insbesondere der dreifach-fluoreszente rekombinante Virus rHSV35R48C22Y, der das Kapsid-Protein VP26 gebunden an ein monomeres red fuoresecent protein (mRFP), das Tegument-Protein VP16 gebunden an ein enhanced cyan fluorescent protein (ECFP) und das Mantel-Protein Glykoprotein H gebunden an ein enhanced yellow fluorescent protein (EYFP) codiert. Es konnte gezeigt werden das dieses rekombinante Virus vollständig replikationskompetent ist, wenn gleich mit einer verzögerten Kinetik, und alle Fusionsproteine in die drei verschiedenen Viruseinheiten integriert. rHSV35R48C22Y weist eine vergleichbare Ultrastruktur wie wild-Typ HSV-1 auf. Die Live-Visualisierung von rHSV35R48C22Y infizierten Zellen liefert neue Erkenntnisse auf dem Gebiet der Dynamik der Kompartimentierung und Interaktion von viralen Proteinen.

Als Extension der vorliegenden Arbeit sowie mit dem Ziel den Prozess der Übertragung der HSV-1-DNA in den Zellkern zu Illustrierung wurde ein Visualisations-System entwickelt, das auf HSV-1 Amplicon Vektoren und der Interaktion des lac-Operator (lacO) mit dem Lac-Repressor (LacR) beruht. HSV-1 Amplicon Vektoren beinhalten lacO Sequenzen, bestehend aus lacR-Bindungsstellen, die in ein rHSV-1 Partikel geschleust wurden, das das VP26 Kapsid-Protein gebunden an ein mRFP enthält (HSV-1 amplicon-35R/lacO). Mit diesen rHSV-1 Partikeln wurden Zellen infiziert die das zellkern-lokalisierte und an ein green fluorescent protein (GFP) gebundene LacR (MRC5/LacR-GFP) stabil expremieren. Die Live-Visualisierung von infizierten Zellen ermöglichte die Verfolgung von HSV-1 Amplicon-35R/lacO Partikeln im Cytoplasma dieser Zellen, der exakte Moment des Eintritts der HSV-1-Amplicon-Vektor-DNA in den Zellkern konnte jedoch nicht erfasst werden. Nichtsdestotrotz konnten leuchtend-grüne Fokusse, die der Bindung von LacR-GFP an lacR-Bindungsstellen des HSV-1 Amplicongenomes entsprechen, im Zellkern von fixierten, infizierten Zellen beobachtet werden. Ebenso konnte demonstriert werden, dass sich diese Fokusse zu Replikations-Kompartimenten entwickeln.

Des Weiteren wurde eine mögliche Anwendung der Einschleusung von fremden Proteinen durch virale Partikel in der vorliegenden Arbeit untersucht. Ein erster Versuchsansatz beinhaltete die Entwicklung eines HSV-1 Vektors, der das Einbringen von therapeutischer Transgen-DNA in die Zielzelle ermöglicht und gleichzeitig enzymatische Aktivität, gebunden an Tegument- und Kapsid-Proteine, aufweist; respektive ein rekombinantes HSV-1 welches das Tegument-Protein VP16 fusioniert mit dem AAV2 Rep Protein codiert. Nicht nur die Expression dieses VP16-Rep-Fusions-Proteins konnte demonstriert werden, sondern auch die Funktionalität dieses Proteins. In einem Replikationsexperiment wurde gezeitg, dass Rep, eingebracht durch das VP16-Rep-Fusions-Protein, in der Lage ist die Replikation einer Transgenkassette, flankiert von AAV2 ITRs, zu unterstützen. In einem zweiten Versuch, wurden genetische Elemente des HSV-1 and AAV2 kombiniert um einen rHAV/AAV hybrid Vektor zu konstruieren, welcher die *in situ* Produktion von rAAV2 Vektoren in rHSV-1 Vektor-infizierten Zellen erlaubt. Die Konstruktion dieses Vektors hatte die Erhöhung der Verbreitung von therapeutischen Transgenen in soliden Hirntumoren zum Ziel.

Introduction

1.1. Viruses

The origin of the word *virus* refers to the Latin "poison". Viruses are obligatory intracellular infectious agents with a vast range of genome sizes and arrangements that leads to a great diversity of genetic cycles and mechanisms of transmission. Viruses, and primarily bacteriophages, are described as the most abundant biological entities and are found in almost every ecosystem on Earth (20, 105). A virus can have a narrow or broad host range, depending on the number of species it can infect, as viruses are non-uniformly distributed among the host taxa (105).

1.1.1. History of virology

The first description of viral infections has been found in a hieroglyph drawn during the ancient Egypt era (1400 B.C.), suggesting a poliomyelitis infection. However, the first reports on the use of inoculation, or variolation, are from India and China around the 8th and 10th century. This practice was brought to the west in the beginning of the 18th century, and improved by Edward Jenner, who first developed a safer vaccination method against smallpox in 1798. The attempts of Louis Pasteur to develop a rabies vaccine (19) lead to the development and improvement, in 1884, by Charles Chamberland, of a filter with pores smaller than bacterias, which was then used by Dmitry Iwanowski (1892) to isolate the tobacco mosaic virus (103, 158, 214). However, the modern concept of viruses as distinct contagious agents ("contagium vivum fluidum") was developed only a few years later, in 1898, by Beijerinck on complementary studies from Iwanowski's work. In the same year Loeffler and Frosch isolated the first animal virus, the agent of food-and-mouth disease (125, 126). The 20th century can also be described as the dawn of virology as many viruses were discovered, and the composition and structure of viruses started to be unveiled. The discovery of bacterial viruses independently by Twort (1915) and d'Hérelle (1917) led to the use of bacteriophages as model systems in the investigation of many aspects of virology. By the end of the 1950's the classical virology is superposed by the development of essential techniques that resulted in advances on understanding the molecular biology of viruses and their host cells. The first complete sequencing of a viral genome in 1976 (233) marks a new era in virology that continues up to the present with increasing improvements on virus knowledge (56, 125).

1.1.2. The origin of viruses

Viruses are thought to be major agents of evolution due to their presence in a broad host and environmental range. Divergent hypotheses on the origin of viruses have been proposed based on different interpretations of the analysis of virus genomes. The main theories of virus evolution can be resumed as: i) The "escaped genes" theory, which suggests that viruses have derived from cellular genes that have switched to the selfish mode of reproduction, is supported by high prevalence of host-related genes (as opposed to virus-specific genes) in many viruses; ii) The "virus world" theory predicts that essential viral genes descended directly from a primordial gene pool that was maintained despite the intense gene transfers occurring at the pre-cellular stage of life's evolution, which explains the spread of these essential genes among apparently unrelated groups of viruses (105); iii) The hypothesis of an independent emergence of viruses within three lineages of RNA-cells evolving from the last universal common ancestor (LUCA) of the known life forms, and mediating the transition to DNA-cells in three independent transitions to DNA genomes, giving rise to archae, bacteria, and eukaryotes (65, 67) - viruses then are suggested to play a major role in the origin of modern DNA genomes, and possibly in the origin of DNA itself due to the diversity of virus-specific proteins involved in DNA metabolism.

The discovery of new viruses, such as the Mimiviruses, brought back the discussion of the origin of viruses reinforcing some old theories, but at the same time, new hypothesis have emerged. The new discoveries have also contributed to a review in the concept of virus. The "virocell" concept has been developed based on the fundamental cellular nature of viruses. According to this concept, viruses are characterized as cellular organisms that comprise a collection of molecular organs (virion, replicon, virion factories) whose integration occurs in the infected cell (7, 35, 66).

1.2. Herpes simplex virus type 1 (HSV-1)

1.2.1. Classification and pathogenesis

Herpesviruses are classified in the *Herpesviridae* family which includes three subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae*, and the *Gammaherpesvirinae*. The *Herpesviridae* is a very successful group as they have a broad host range and are able to infect a wide variety of tissues within their hosts.

Herpes simplex virus type 1 (HSV-1) belongs to the *Alphaherpesvirinae* and is a common human pathogen present in 65 % to 90 % of the human population (32). Infections with HSV-1 result in mucocutaneous vesicular eruptions, and, in some cases, can lead to sporadic encephalitis. However,

asymptomatic infections can also occur resulting in the widespread transmission of the virus (100). After a primary infection, HSV-1 establishes latency at the ganglionic neurons present at the site of primary infection, predominantly the trigeminal ganglia for orofacial infections, supporting a permanent infection in the host. Upon stimulation, the latent HSV-1 virus can reactivate, resulting in a reoccurrence of symptoms at the site of initial infection (34).

1.2.2. The structure of the HSV-1 particle

The herpes simplex virus type 1 (HSV-1) virion is composed of a double stranded 152 kbp-DNA core protected by a 100 nm-diameter icosahedral capsid, around which the tegument, a proteic layer is limited by the outer lipoproteic envelope (103) (Fig. 1).

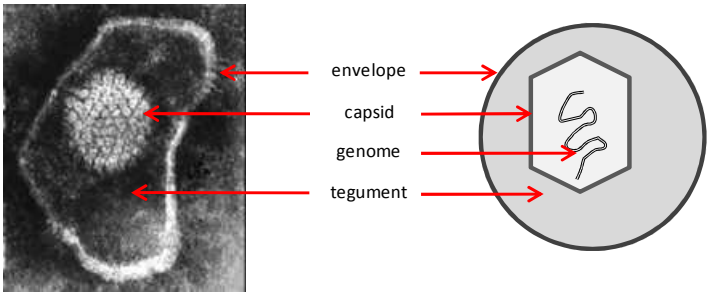

Figure 1. Structure of the HSV-1 particle. The herpes simplex virus type 1 (HSV-1) virion is composed of a double stranded DNA core protected by an icosahedral capsid of 100 nm-diameter, around which the tegument is limited by the outer lipoproteic envelope. The diameter of the enveloped HSV-1 particle ranges from 200-300 nm. Electronmicrograph from Linda Stannard, of the Department of Medical Microbiology, University of Cape Town - http://www.virology.net/Big_Virology/BVHomePage

The HSV-1 genome

The HSV-1 genome expresses at least 87 genes oriented in both directions (forward and reverse). Structurally, the genome can be divided into unique long (UL) and unique short (US) segments, connected by regions of inverted repeats, the internal repeats (IR_L and IR_S) and the terminal repeats (TR_L and TR_S) (Fig. 2). In addition, at both ends, the genome has a relatively short redundant sequence, which may be directly repeated several times (103). The HSV-1 genome undergoes inversions that result from recombination events mediated by the viral DNA replication machinery, yielding four genomic isomers in equimolar amounts (215). The HSV-1 genome contains three origins of DNA replication: ori_L, located within UL, and ori_S, which is found within TR_s and is therefore duplicated (Fig. 2) (198). The localization of these origins suggest a temporal activity role, although redundancy has also been observed since mutant viruses with a single class of origin show no defect

in replication (93). DNA cleavage/packaging signals (*pac*) are present at both termini of the genome as well as at the junction between the long and the short segments (Fig. 2). Of note, *ori* and *pac* are the sole *cis*-acting elements necessary for HSV-1 DNA replication and packaging (46, 45, 182, 196).

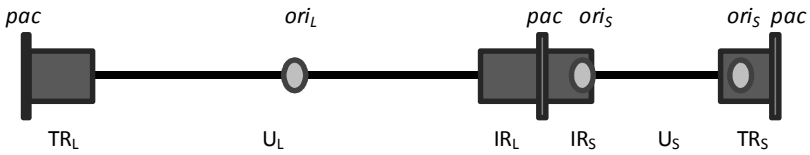

Figure 2. Schematic map of the HSV-1 genome. The HSV-1 genome is a linear double stranded DNA of approximately 152 kbp in size, composed of two unique segments, U_L and U_S, which are flanked by repeated regions, TR_L /IR_L and TR_S/IR_S, respectively. The minimal *cis* elements required for HSV-1 DNA replication and packaging are the origin of DNA replication, *ori,* and the packaging, *pac,* signals.

The HSV-1 capsid

The HSV-1 capsid is composed of seven virion proteins (VP), encoded mostly by genes present in the unique long (UL) segment of the genome: VP5 (UL19), VP19C (UL38), VP21 (UL26), VP22a (UL26.5), VP23 (UL18), VP24 (UL26), VP26 (UL35), UL6, and UL25 (103, 231). Viral capsids play important roles in the protection, transport and delivery of the HSV-1 genome into the host cell nucleus. After the onset of viral replication and protein synthesis, capsid proteins are also involved in the maturation of the capsid structure and packaging of the viral genome (11, 87, 154, 153, 204, 205). The icosahedral vertices of the capsid shell are composed of arrangements of the VP5 capsid protein forming hexons (150) and pentons (12) that are connected by a complex formed with two copies of VP23 and one copy of VP19C (181, 229). Six copies of VP26, a 12 KDa polypeptide encoded by the UL35 gene, occupy the tips of the hexons (136, 232). UL35 is a late gene, which is expressed after the onset of DNA replication and has been shown to have multiple phosphorylated forms (137). Although not essential for viral replication in tissue culture (33, 48), deletion of the UL35 gene or fusion of VP26 with fluorescent proteins have been shown to impair recombinant HSV-1 growth (3, 48, 53).

The HSV-1 tegument

The HSV-1 tegument consists of several VPs and infected cell proteins (ICPs), encoded by genes present in both unique long (UL) and unique short (US) segments of the HSV-1 genome: VP1/2 (UL36), VP11/12 (UL46), VP13/14 (UL47), VP16 (UL48), VP22 (UL49), ICP0, ICP4, ICP34.5, virus-host shut off protein (vhs) (UL41), US2, US3, US9, US10, US11, UL11, UL13, UL14, UL16, UL20, UL21, UL37,

UL51 and UL56. Recently, HSV-1 proteins encoded by the genes UL7, UL23 (thymidine kinase), UL50 (dUTPase), and UL55 were described as most probably being located in the tegument (120) ((101). Tegument proteins play different roles in the viral life cycle: they are involved in transport of viral capsids to the nuclear pores, through recruitment of cellular molecular motors during entry and egress (54, 120, 123, 194), attachment to the nuclear pore complex and release of viral DNA from the capsid into the nucleus (VP1/2) (39, 97), remodeling of the host cell environment by direct delivery of virion proteins into the cell and/or by *de novo* expression of viral proteins (60), regulation of host cell gene expression (10, 55, 119), and distribution and assembly of viral components during infection (101, 120).

The most abundant tegument proteins VP1/2, VP11/12, VP13/14, VP16, and VP22 play also a structural role in virion composition, whereas ICP0 and ICP4, which are present in relative low abundance, are involved in regulatory roles and are carried in the tegument rather than being structural (113, 146). The identification of interactions among HSV-1 tegument proteins and the analysis of cellular localization of these proteins (21, 209), together with structural and biochemical studies (80), provide evidence for an ordered addition of tegument proteins during assembly (140). Despite the large number of tegument proteins, the essential ones for viral growth in cell culture are VP1/2, UL37, VP16, and ICP4 (101). The major structural component of the virion tegument is VP16 a 54 KDa protein encoded by UL48 gene (217). VP16 is an alpha-trans inducing factor (α-TIF), responsible for transcriptional regulation of immediate-early genes and also involved in the modulation of the activities of other viral components (29, 160). Due to its interaction with other viral proteins, VP16 is absolutely required for assembly and packaging of infectious virus (104, 217); moreover, it plays essential roles in viral maturation and egress (70, 147).

The HSV-1 Envelope

Despite some controversy on the composition of the envelope, basically it is a lipid bilayer formed by i) glycoproteins (g) gB, gC, gD, gE, gI, gH, gJ, gK, gL, gM, gN, gO; ii) non-glycosylated membrane proteins encoded by the genes UL20, UL24, UL34, UL45; and iii) two not-well characterized membrane proteins (UL43, UL49.5) (28, 120). The glycoproteins are the major antigenic determinants for the host-specific recognition, and they are involved in cell entry, cell-to-cell spread, and immune evasion. The glycoproteins gB, gD, and the gH-gL complex are essential for HSV-1 replication in cell culture, cell fusion, and cell-to-cell spread (5, 23, 168). gC participates as a receptor for the C3b factor of the complement system, acting in the immune evasion mechanism (129). The

glycoproteins gE and gI form a complex required for efficient cell-to-cell spread, especially between cells that form extensive cell junctions (51, 61).

Glycoprotein H (gH) is a 110 kDa glycoprotein, product of the UL22 gene, required for virion infectivity, and membrane fusion, but not for receptor binding (64). However, gH must be coexpressed with gL, a 224 amino acid protein, in order for both proteins to be properly processed, folded, and transported to virion envelope and infected cell surface. In absence of gL, gH is retained in the endoplasmatic reticulum (ER) and is not incorporated in the viral envelope (92, 180). Therefore, the gH/gL complex plays essential roles in viral penetration, cell-to-cell spread, and syncitium formation (180). Both glycoproteins are conserved in other members of the herpesviruses, confirming the essential role of the gH/gL heterocomplex during infection, although some differences on assembly, structure, and intracellular transport of the heterocomplex exist between individual herpesvirus (102, 163).

1.2.3. The HSV-1 Life Cycle

Entry

HSV-1 enters the host cell essentially through three steps: attachment, stabilization of the attachment, and penetration (Fig. 3). However, depending on the cell type, different entry pathways have been described: via direct fusion with the plasma membrane (197), via endocytosis followed by fusion within an acidic endosome (73, 156, 177), by fusion within a neutral endosome (144), or via phagocytosis-like uptake (1, 36, 132).

Initially, HSV-1 attaches to the cell membrane by independent binding of gC and/or gB to heparin sulphate proteoglycan (HSPG) (190), suggesting a superposition of glycoprotein functions. The attachment is stabilized through exclusive binding of gD to one of several herpesvirus entry mediator protein receptors (Hve): i) Hve A, a member of the tumor necrosis factor α receptor family (HVEM= TNFRSF14); ii) Hve B, Hve C (nectin-1 α), and HIgR (nectin-2α), members of the immunoglobulin family; and iii) 3-O-sulfated heparin sulfate (177). Conformational changes on gD activates the fusion machinery: an activation signal is transmitted to gB and gH/gL to assemble with gD and form a fusogenic complex (5, 23, 177). Association of gB with lipid rafts domains in the cell membrane after attachment and during entry have been reported suggesting the existence of a gB receptor present in these domains rich in cholesterol (12). More recently, it has been found that the paired immunoglobulin-like type 2 receptor α (PIRL α) associates with gB and functions as an entry co-receptor for HSV-1 infection (186).

Cytoplasmic transport of HSV-1 particles

After penetration, nucleocapsids are transported towards the nucleus mostly by the tegument proteins that remain associated to the capsids, such as UL36, UL37 and VP11/12 (39, 120, 148). HSV-1 tegument proteins have been demonstrated to dissociate from the nucleocapsids after phosphorylation of tegument proteins (VP12/13 and VP16) (71) by both viral (UL13 and US3) and cellular kinases early after infection (132, 146). The described interaction of the VP26 capsid protein with dynein has been suggested to play a role in the retrograde transport in non-polarized cells (4, 54, 194, 223). Although no direct interaction has been described, the inner tegument proteins (UL36, and UL37) are likely to bind dynein chains of the microtubule-dependent minus-end-directed motor complex dynein-dynactin (Fig. 3) (3, 223, 230).

Nuclear entry of the HSV-1 genome

Upon arrival at the nuclear membrane, the docking of nucleocapsids at the nuclear pore complex (NPC) is mediated by interactions of viral tegument (UL36) and capsid (UL25, UL17) proteins (37, 47, 97) with nucleoporins importin-β (161, 194), CAN/Nup214, hCG1, and Nup358 (Fig. 3) (39, 165). In addition, UL25 has been shown to bind to the UL36 tegument protein and to the portal UL6 capsid protein (30), thus acting as a key linker between nuclear pore binding and viral DNA release processes (97, 165, 171). As soon as the nucleocapsid docks at the NPC, a mechanism involving the proteolytic cleavage of VP1/2, and subsequent conformational changes in the capsid pentons, allows the release of the HSV-1 DNA into the nucleus via the UL6 portal protein (97, 230). The large HSV-1 genome is densely packaged within the capsid and seems to be free of bound histones or other proteins (18, 230). Import of the genome in a linear form, by passive diffusion has been predicted to take over 30 min, rendering the genome accessible to degradation (183). However, studies using atomic force microscopy demonstrated that instead, the HSV-1 genome is delivered as a highly condensed, rod-like structure through the widening of the NPC central channel (189). The HSV-1 genomes were observed as subviral components of 34-40 nm-diameter and length of 130-160 nm at the cytoplasmic side of the nuclear envelope, and were translocated selectively towards the nucleoplasmic side (189). The translocation process is slow and the extruded genome remains associated with the cytoplasmic side of the NPC. In contrast, the empty capsid is quickly dissociated from the NPC once the genome is released (194). These mechanisms may also involve viral proteins as well as cellular factors that can direct the nuclear import of the genome.

Regulation of viral gene expression

HSV-1 can establish a lytic or latent infection depending on the cell type infected and on the cellular and viral factors present at the time of infection (Fig. 3). Lytic infection occurs mostly in epithelial cells through the activation of a transcription complex mediated by the VP16 tegument protein, followed by a temporally regulated cascade of viral gene transcription. Thus, HSV-1 genes are classified as (i) immediate-early (IE) or α-genes; (ii) early (E) or β-genes; (iii) leaky-late, or γ-1 genes; and (iv) true-late or γ-2 genes (103). During the first steps of HSV-1 infection, VP16 dissociates from the capsid and interacts with the host cell factor (HCF-1), an essential cellular transcriptional co-activator and a component of multiple chromatin modification complexes (108, 167, 187). Then the VP16-HCF-1 complex is primed for association with the cellular octamer DNA-binding transcription factor (Oct-1) (225). The transcription complex binds via Oct-1 on regulatory elements present in each IE-gene promoter, whereas HCF-1 recruits transcription factors and the chromatin modulation machinery, contributing to enhance the efficiency of IE gene transcription (91, 151). Although VP16 seems to play a role in transcription of IE genes via interactions with specific sequence motifs of IE promoters (179), the co-activator factors recruited by the transcription activation domain of VP16 (histone acetyltranferases, p300 and CBP; and the ATP-dependent chromatin remodeling enzymes (BRM, Brg-1)) have been reported as non-essential for IE gene expression during lytic infection *in vitro* (110).

The IE protein synthesis peaks around 2-4 h post infection (p.i.), and these proteins have mainly regulatory functions (103). For instance, IE proteins activate transcription of E and L genes by a coordinated interaction with cellular transcription factors (SP1, CTF, and USF; TFIID/TBP) and binding to specific E, and later, L promoters (79, 176). IE proteins are also responsible for downregulating the expression of IE genes at late stages of virus replication (109, 207). Transition from the IE to E viral gene expression seems also to depend on dissociation of the histone deacetylase from the CoREST/REST repressor complex by ICP0 (81, 179).

The E genes encode some of the tegument proteins and envelope glycoproteins, as well as enzymes required for DNA metabolism, including thymidine kinase (Tk), ribonucleotide reductase, ICP8, DNA helicase-primase, origin-binding protein and DNA polymerase. The synthesis of these proteins reaches a peak at 5-7 h p.i., and can be detected as early as 3 h p.i.. These enzymes signal the onset of viral DNA replication which in turn leads to the induction of late gene expression (16). ICP27 also plays a role on regulating the switch from early to late gene expression, through the repression of the expression of certain IE (ICP0, ICP4) (164, 228), and E (ICP8) genes (162). In contrast to the leaky-late genes that contain an SP1 binding site, the true-late genes have no regulatory elements

upstream of the TATA-box sequence, thus depending on viral DNA replication for their expression. The late genes encode most of the structural components of the virion and are important for the assembly of virus particles (103).

After infecting and replicating in epithelial cells, HSV-1 virions enter the termini of sensory neurons, which innervate the site of the primary infection, and reach the nucleus of the neurons via anterograde transport. Establishment of latency is another mechanism of HSV-1 infection that is restricted to a specific sub-type of sensory neurons that are recognized via interactions among neuronal proteins and elements of the latency-associated transcript (LAT) region on the HSV-1 genome (15). During latency, the genome is thought to be in a transcriptionally silent state, with only the LAT gene being abundantly transcribed (139). HSV-1 LAT has been associated with an increased efficiency of latency establishment, reactivation, and block of apoptosis (15). LAT has been described to function as a primary microRNA (miRNA) precursor that encodes at least four miRNAs in HSV-1 infected cells; two of which are likely to be involved in post-transcriptional regulation of gene expression, for instance the ICP0 and ICP4 IE genes (206).

Eventually, the silent HSV-1 genome can be reactivated resulting in lytic infection and recurrence of clinical signs at the site of primary infection. Regulation between latency and reactivation of the HSV-1 genome appears to be finely controlled by multiple regulatory elements at an epigenetic level (15). The reactivation mechanism seems to be triggered by stress stimuli, or UV exposure, leading to modification on the chromatin state (167). It has been hypothesized that the HCF-1 modification complexes may play a central role in this process, in a manner analogous to its role in the initiation of lytic infection. Recently, *de novo* synthesis of HSV-1 VP16 in neurons has also been shown to initiate expression of viral IE genes with resulting reactivation from latency in the nervous system (203).

HSV-1 DNA Replication

Within hours after infection, the incoming linear virion genome has been described to circularize (49). Although the mechanism has not been well established, one model predicts that circularization may involve recombination of the terminal repeats, resulting in a teta-replication mode for the initial round of replication (178), providing the template for a rolling circle mode of replication (191). However, recent studies revealed that the HSV-1 DNA circles are formed only in cells infected with viruses that do not express ICP0, or in the complete absence of viral gene expression, conditions that mimic the latent state of the virus (Fig. 3). Evidence has also been provided that genome circularization is unlikely to be required during productive infection. These data, coupled with the fact that teta intermediates have not been detected *in vivo* or *in vitro* (114), indicate that other

models for HSV-1 DNA replication will have to be considered, such as recombination events that result in endless genomes (94).

HSV-1 genome replication initiates at the viral origins of DNA replication (ori_S and ori_L) at 3-4 h p.i., reaching maximum efficiency between 8-16 h p.i.. Nuclear components engage an ordered rearrangement process, forming globular domains called replication compartments (RC), which are sites of viral DNA replication, cleavage, and packaging (131, 195). The viral replication machinery is composed of ICP8 (UL29), which is a single stranded-DNA binding protein and an essential replication factor; a heterodimeric polymerase complex (UL30-UL42); the helicase-primase complex (UL5, UL8, UL52); and an origin binding protein (UL9) that can recognize and bind specifically to ori_S and at low affinity also to ori_L (27, 116, 121, 169). In addition, some tegument proteins also play important roles in the regulation of viral replication. For instance, the virion host shut-off (vhs) protein (UL41) is an endoribonuclease that degrades mRNA soon after dissociating from incoming capsids. The degradation of mRNA may be required for rapid turnover of viral mRNA and for immune evasion (52). UL23, a thymidine kinase, and UL50, a dUTPase enzyme, have key roles in nucleotide metabolism during infection of non-dividing host cells (63, 174, 174). UL36 plays important roles in protein turnover, membrane trafficking, and transcription due to its deubiquitinating enzymatic activity that may act in the direct regulation of the cellular post-translational ubiquitination process (99). UL49 interacts directly with histones or with the cellular histone chaperone template-activating factor I (TAF-I) and appears to regulate nucleosome formation (208). The interaction between HSV-1 ICP34.5 and proliferating cellular nuclear antigen (PCNA) has also been shown to regulate viral DNA replication (82). It has been reported that some cellular proteins are also involved in viral DNA replication, such as proliferating cell nuclear antigen (PCNA), single stranded-DNA binding protein (RPA), retinoblastoma protein (Rb), and p53 (44, 219).

The hypothesis that replication of HSV-1 depends on cellular S-phase induction is supported by the observation that HSV-1 replicates more efficiently in replicating than in growth-arrested cells, and by the association of HSV-1 replication with cellular functions known to be involved in cell cycle progression. For instance, ICP0 and VP16 seem to be involved in the induction or replacement of cellular activities normally activated in a cell-cycle regulated manner (187). ICP4, ICP8 and TK transcripts were shown to be inhibited in the presence of cdks inhibitors as well as viral replication (187). HCF-1, an important regulator of cell cycle progression (77, 222), has been recently described to play a role in HSV-1 replication, through the coupling of the histone chaperone Asf1b to viral DNA replication components (167).

HSV-1 Egress

Different models of HSV-1 maturation, envelopment and egress have been proposed. The most accepted model, the envelopment-deenvelopment-reenvelopment, suggests that viruses acquire some tegument proteins and a primary envelope when contacting the inner nuclear membrane and budding into the perinuclear space. The perinuclear virions then fuse with the outer nuclear envelope, and unenveloped nucleocapsids are released in the cytoplasm where they acquire the inner and outer tegument proteins before a secondary envelopment (Fig. 3) (141, 142).

Another model proposes two distinct pathways: the first pathway involves envelopment at the inner nuclear membrane followed by intraluminal transport of perinuclear enveloped nucleocapsids through a continuum of outer nuclear membrane and rough endoplasmic reticulum (RER), passing through the Golgi, where transport vacuoles are formed (115). Alternatively, capsids leave the nucleus via impaired nuclear pores and are enveloped at the cytoplasmic membranes of RER or Golgi (115, 220, 221). In all models, assembled virions are described to follow the secretory pathway, being recruited into vesicles and released by exocytosis (Fig. 3).

Figure 3. The HSV-1 life cycle. During HSV-1 infection, attachment of glycoproteins gC and gB to heparin sulphate proteoglycan (HSPG) at the host cell plasma membrane is stabilized by binding of the gD to a specific herpesvirus entry mediator receptor (Hve). Depending on the cell type, entry of HSV-1 can occur via distinct pathways: fusion or endocytosis. Fusion of the HSV-1 envelope with the plasma membrane leads to the release of a naked nucleocapsid into the cytoplasm that is transported via microtubules to the nucleus. Endocytosis of the HSV-1 particle may be dependent on low endosomal pH, leading to the escape of the nucleocapsid from the endocytic vesicles. Docking of the nucleocapsid to the nuclear pore complex (NPC) is followed by release of the HSV-1 genome into the cell nucleus. There, it can lead to a latent infection, where the viral genome is maintained in an episomal form, or lead to a lytic infection. The lytic cycle involves the transcription and replication of the HSV-1 genome, cleavage of concatemeric genomes into unit-length genomes and assembly of nucleocapsids inside the nucleus. Although still a matter of debate, tegumentation and envelopment are described to occur during exit of the nucleus and transport to the Golgi compartment. Here, the most conventional envelopment model is shown: capsids leave the nucleus by budding with the inner nuclear membrane, then perinuclear enveloped capsids undergo de-envelopment by fusion with the outer nuclear membrane releasing unenveloped capsids into the cytoplasm where they acquire the final envelope by budding into the trans-Golgi network (TGN). HSV-1 virions exit the cell via transport vesicles that fuse with the plasma membrane releasing a mature enveloped virion.

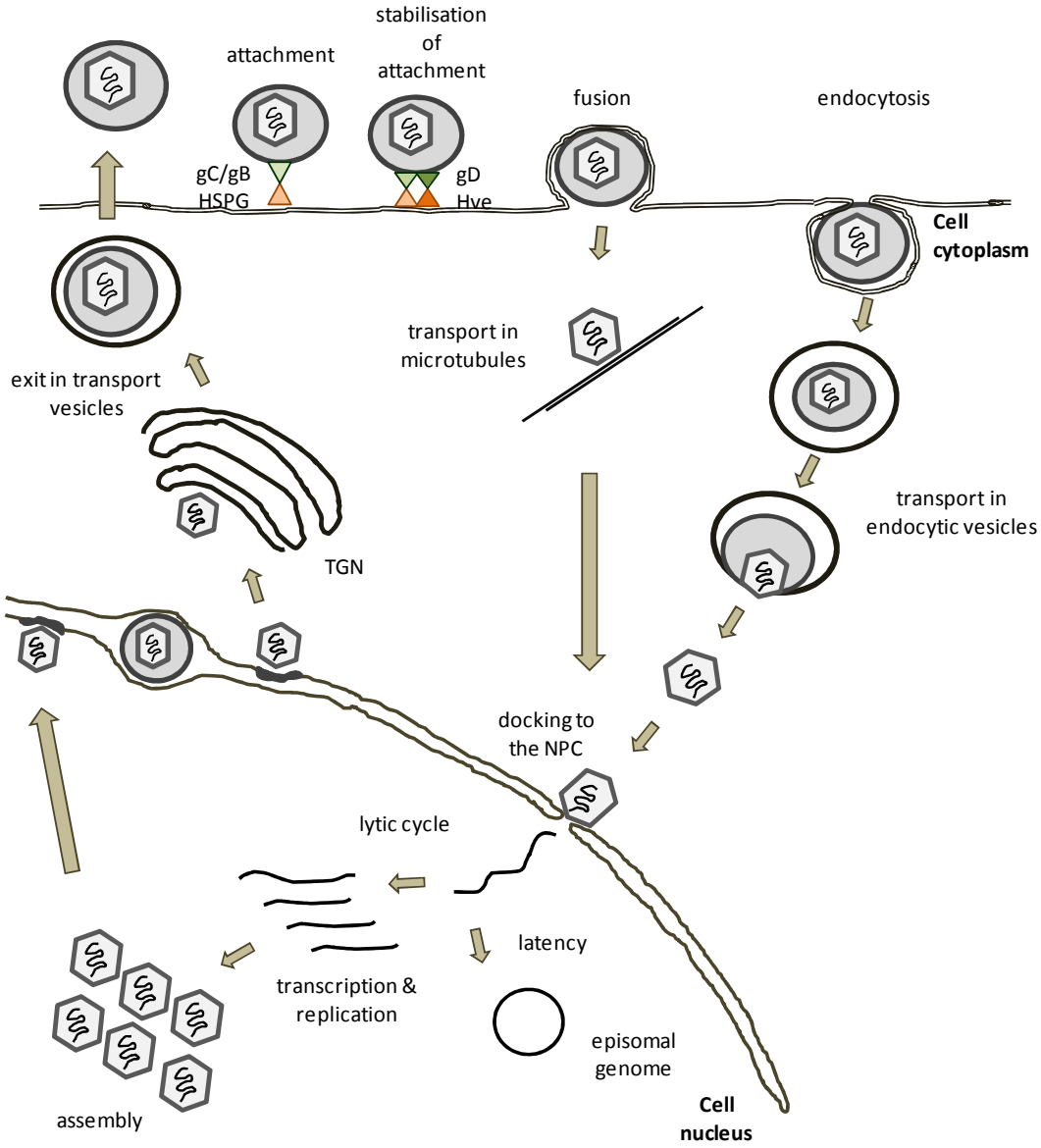

Figure 3. The HSV-1 life cycle. See previous page for legend.

1.3. Adeno-associated virus (AAV)

Adeno-associated viruses (AAVs) belong to the genus *Dependovirus* within the subfamily *Parvovirinae* of the family *Parvoviridae* (103). AAVs are non-enveloped viruses that contain a small genome inside an icosahedral capsid (Fig. 4A). AAVs are replication-defective viruses that depend on helper viruses and are common contaminants on Adenovirus cultures. Different AAV serotypes have been identified that can infect a broad range of species, such as the primate AAV serotypes 2, 3, 4, and 5 (8, 59). Despite being widespread among species and infecting different tissues, AAV infections have not been associated with any pathology. Primate AAV serotypes share significant sequence similarities, and the occurrence of cross-reaction of neutralizing antibodies may be species specific or depend on tissue type or route of administration (166, 226).

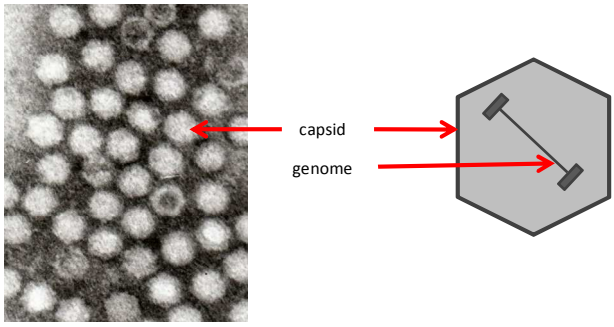

Figure 4. The AAV particle. (A) Structure of AAV viral particle**.** AAV contains a single stranded DNA genome of 4.7 kb protected by an icosahedral capsid of approximately 20 nm-diameter. Electronmicrograph from Graham Colm Original uploader was GrahamColm at en. wikipedia Permission:CC-BY-SA; Released under the GNU Free Documentation License.

1.3.1. The AAV2 genome

The genome of the AAV prototype serotype 2 (AAV2) is a linear single stranded DNA of 4.7 kb, and either the positive or negative strand can be packaged with equal efficiency. The genome is flanked by inverted terminal repeats (ITR) of 145 nucleotides, a palindromic sequence that forms a hairpin secondary structure and plays important roles in the replication and packaging of the AAV2 genome (155). The ITRs flank two open reading frames (ORFs), *rep* and *cap* that encode overlapping proteins through alternative splicing. The Rep proteins, Rep78/68 and Rep52/40 are transcribed from two different promoters, p5 and p19, respectively, and are involved in DNA replication, transcription, and chromosomal integration (Fig. 5A). The regulatory activity of Rep seems to include the maintenance of a constant ratio of Rep and Cap proteins during infection in order to keep the balance between AAV2 genome replication and packaging. The Cap ORF encodes three overlapping proteins, VP1, VP2

and VP3, from a single promoter, p40. These three structural proteins together form the icosahedral capsid, which has a diameter of 18 to 26 nm (103).

1.3.2. AAV2 replication

AAV2 is a replication defective virus as it depends on a helper virus, such as an adenovirus (88), a herpesvirus (24, 138), vaccinia virus (188), or papillomavirus (86) for productive replication. Helper viruses are also responsible for inducing a cell cycle arrest in late S or G2 phase, as in the case of adenoviruses (78) or for down regulating host cell functions as in the case of HSV-1 as the helpervirus (202). Many studies have assessed the different elements from the helper viruses required for AAV2 replication. A model has been proposed where the HSV-1 helicase/primase proteins constitute a scaffold that recruits ICP8, Rep and cellular replication proteins (E.g. RPA) to the self-primed AAV2 DNA in replication compartments (83, 192, 199, 216). The HSV-1 polymerase complex is used together with the cellular machinery for AAV2 replication (2, 212). Interestingly, an inhibitory effect of Rep78/68 proteins on HSV-1 replication has been described (75, 84), suggesting a regulatory effect of AAV2 over HSV-1. The p5 promoter sequence has been shown to contain two signals, the Rep binding site (RBS) and the terminal resolution site (TRS) (69, 111), that can act as an alternative origin of DNA replication in the presence of adenovirus (149, 157) or HSV-1 helpervirus functions (74) (Fig. 5B).

AAV2 genome replication is based on a “rolling hairpin model”. The hairpin structure at the ITR acts as a primer that converts the DNA into a double-stranded template, and together with the essential *cis*-acting elements RBS and TRS, and helpervirus functions, the replication and transcription of the AAV2 genome is initiated (133, 212). The Rep78/68 proteins play major roles in the replication process due to DNA-binding, endonuclease, and helicase activities. Rep binds to the RBS inducing a site- and strand-specific nick at the TRS, thus creating a new genome end and allowing the reinitiation of the synthesis of a monomer extended form that can be packaged (213). Double-stranded dimer molecules can be formed if the hairpin structure in the monomer is not resolved before reinitiation on the other genome end (89).

In the absence of a helper virus, the AAV2 genome can integrate into a specific site termed AAVS1 on chromosome 19q13.3-qter of human cells (106, 185, 201, 218). The integration is mediated by Rep78/68 and ITRs through a non-homologous deletion/insertion recombination event (185) ((193, 218, 227). Also, an integration efficiency element (IEE) has been identified within the p5 promoter of AAV2 (170), and more specifically a 16-bp RBS was shown to be sufficient for AAV2 genome integration (62). Rescue of the integrated AAV2 genome is possible by superinfection with a helper

virus (2). Although HSV-1 ICP0 seems to contribute to the activation of the *rep* gene from latent AAV2 genomes (72), it is not sufficient to induce Rep synthesis (2). Some studies have demonstrated the autonomous replication of AAV2 under special conditions (143); however, the efficiency of replication is significantly lower than in presence of a helper virus.

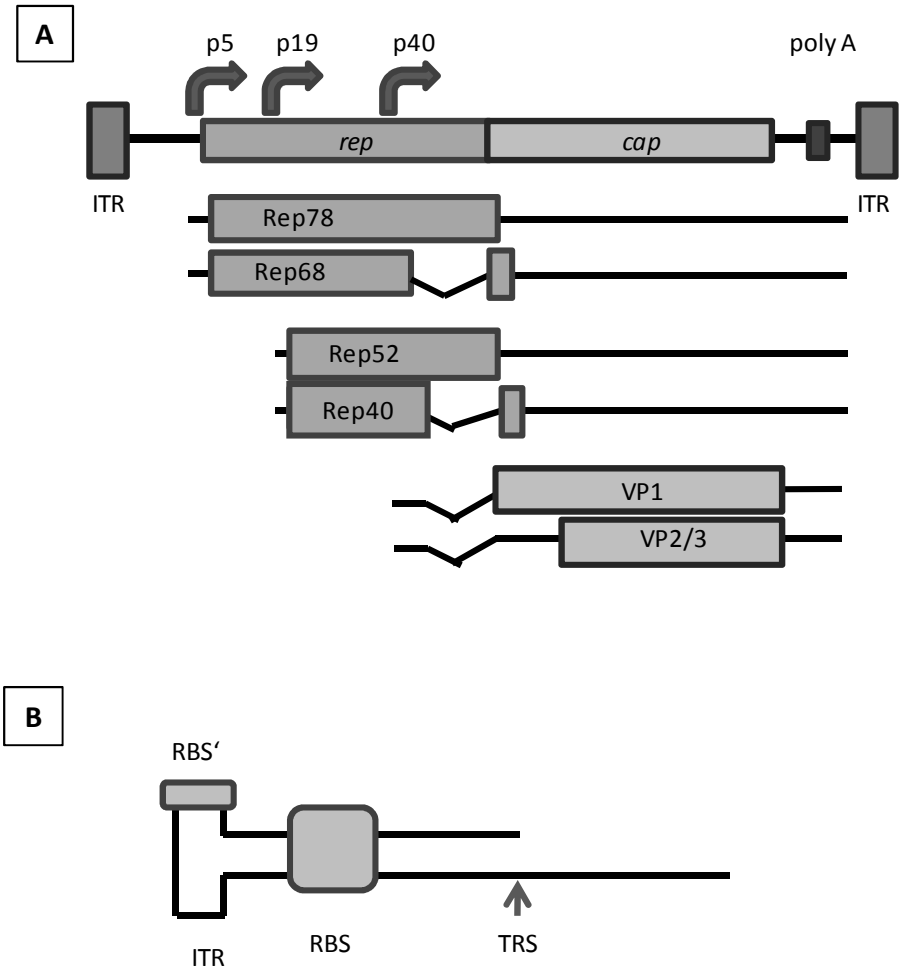

Figure 5. **Schematic map of the wild type AAV2 genome.** (A) The genome of AAV2 is composed of two open reading frames encoding the *rep* and *cap* genes from different promoters (p5, p19, p40) and alternative splicing. (B) Secondary structure formed by the inverted terminal repeat, ITR. Depicted are the Rep binding sites, RBS, and the terminal resolution site, TRS.

1.3.3. AAV2 infection

AAV2 can infect different tissues and bind to unique cellular receptors, which can account for a serotype-specific tissue tropism. Several cellular receptors used by AAV2 for cell entry have been identified, including heparan sulfate (9), fibroblast growth factor receptor (175), and integrin alphaVbeta5 (200). The initial steps of AAV2 infection, attachment to cellular glycosaminoglycan receptors and interactions with coreceptors seem to define the intracellular trafficking pathway of the capsid. Upon entry, AAV2 capsids are endocytosed via clathrin-coated pits (50), and transported through both late and recycling endosomes. Trafficking in recycling endosomes appears to be favorable for efficient transgene expression (122). The process of uncoating is still not well characterized (50), however, AAV appears to enter the nucleus through a mechanism independent of the nuclear pore complex (43).

1.4. HSV-1 and AAV based vector systems

Many studies have been focusing on the use of gene therapy for treatment of inherited or acquired diseases. Different vectors have been employed in gene therapy with most of them being modified viruses. The use of specific viruses as vectors for gene therapy relies on their efficient capacity of transducing different types of cells with foreign therapeutic genes. The specific characteristics of each virus confer the possibility for a broad range of applications. Encouraging results from pre-clinical and clinical studies are leading to improvements in the development of safer and more efficient viral vectors.

1.4.1. HSV-1 based vector systems

The advantages of HSV-1 for its use as a viral vector include: (i) a large transgene capacity; (ii) high transduction efficiency and broad cell tropism, including dividing and non-dividing cells; and (iii) ability to establish latency. On the other hand, HSV-1 is a human pathogen which could consequently lead to (i) host immune responses; (ii) cytopathogenic effects; and (iii) reactivation or recombination events with latent wild type HSV-1 (95). Two different HSV-1-based vector systems have been developed: recombinant HSV-1 vectors and HSV-1 amplicon vectors.

Recombinant HSV-1 vectors

Recombinant HSV-1 vectors are based on HSV-1 mutants that initially have been used for the functional study of genes and development of vaccines. Two different approaches have been developed for the use of recombinant HSV-1 vectors (128). The replication-conditional vectors are attenuated viruses in which genes non-essential for replication *in vitro* are either mutated or deleted; such vectors may be suitable for therapeutic treatment of tumors. Replication-conditional vectors have also been under investigation for application in disorders of the central nervous system (CNS), such as lysosomal disorder (13), multiple sclerosis (22) or acute injury (112). Candidate genes encoding thymidine kinase (TK), ribonuclease reductase (RR), the virion-host shut off (vhs) and the ICP34.5 proteins have been studied in order to eliminate some of the HSV-1 drawbacks (40, 145, 184, 210). Among these, the HSV γ_1 34.5 gene is essential for HSV-1 pathogenicity, although not essential for replication *in vitro*. In the absence of both copies of this gene, HSV-1 is described as a safe oncolytic vector for gene therapy as it is able to replicate in mitotically active tumor cells but lack this capability in postmitotic cells, such as neurons (31, 42, 90, 117).

Replication-defective vectors are constructed by deletions in genes essential for the lytic cycle, for instance the IE genes (14, 26, 107). Replication-defective vectors are mostly applied for gene replacement therapy (41), and have been evaluated as vectors in animal models of neuropathies, such as epilepsy, multiple sclerosis, Alzheimer's disease, Parkinson's disease, chronic pain, and lysosomal storage disorders (76, 128, 130, 152, 173).

Despite of the promising results of preclinical studies, some obstacles have still to be overcome as replication-defective mutants of HSV-1 can cause cytopathic effects in primary cultures of neuronal cells and inflammatory responses in neural tissue *in vivo*; and because most viral and non-viral promoters are silenced after injection into the brain. The development of new HSV-1 vectors have been focusing in achieving nontoxic, long-term gene expression in neurons, and improving target expression to specific neuronal populations.

HSV-1 amplicon vectors

HSV-1 amplicons are defective, helper-dependent vectors in which the HSV-1 genome has been replaced by an amplicon plasmid containing the minimal HSV-1 elements *ori* and *pac*. In the presence of a helper virus genome, the amplicon genome containing the transgene of interest is replicated and packaged into HSV-1 particles. The use of HSV-1 amplicon vectors for gene therapy allows the safe delivery of up to 150 kbp of foreign DNA. The absence of the replication machinery in HSV-1

amplicon vectors renders these vectors suitable for transduction into quiescent cells such as neurons, muscle cells, and hepatocytes (57, 58).

1.4.2. AAV vectors

AAV2 vectors are among the most widely used recombinant virus vector in gene therapy. This is due to the characteristics of AAV2 that include a broad cell tropism, lack of pathogenicity, and stable long-term gene expression. For the construction of recombinant AAV vectors, the Rep and Cap ORFS are replaced by a transgene cassette flanked by the ITRs. Replication of the vector genome and packaging into AAV capsids supported by Rep, Cap, and helper functions provided *in trans* (201). Preclinical studies have been performed with rAAV2 vectors for different diseases (124, 134, 172). The high efficiency in transducing distinct regions of the brain by the specific AAV serotypes together with the long term expression of transgenes in the CNS has increased the interests in developing rAAV vectors for treatment of neurological diseases (25, 98, 127). Improvements in the construction of rAAV vectors have focused on the regulation of transgene expression to confer safety, enhancement of the transduction efficiency, and evasion of antibody neutralization (135, 150) ((224).

1.4.3. HSV/AAV hybrid vectors

The combination of advantageous viral properties that confer enhancement of transgene delivery, vector stability, and long-term transgene expression, led to the development of hybrid vectors (106, 159). In order to take advantage of the specific characteristics of HSV-1 and AAV, different strategies were employed for the construction of HSV/AAV hybrid vectors. One of the drawbacks in using AAV as a vector for gene therapy is the difficulty in scaling-up the production of rAAV vectors. To improve production, replication defective rHSV-1 have been designed for use as helper virus, and for the delivery of AAV *rep* and *cap* genes, generating higher yields of rAAV free of helper virus contamination (17, 38).

HSV/AAV hybrid amplicon vectors have been developed which, in addition to the HSV-1 amplicon elements, incorporate the AAV *rep* gene and a transgene cassette flanked by the AAV ITRs. This strategy conserves the high efficiency of gene transfer and the large transgene capacity from HSV-1, as the hybrid vector genome is packaged into HSV-1 particles, while allowing site-specific integration of the ITR-flanked transgene cassette into the AAVS1 sequence of human chromosome 19 (85). Because the AAV *rep* gene is placed outside of the ITR cassette it eliminates the possibility for rescue/excision of the integrated ITR cassette in the case that the cell is infected by a helper virus. HSV/AAV hybrid vectors can be packaged into HSV-1 virions by using either helper virus-dependent

or helper virus-free packaging systems (68, 96). Studies with these vectors demonstrated long-term expression and site-specific integration of the transgene cassette (85, 211). However, a decrease on the titers of HSV/AAV hybrid vectors has also been observed which was likely due to inhibitory effects of Rep on the HSV-1 replication machinery (6, 84, 85, 211). In order to overcome this problem, a study showed an increase in HSV/AAV titers when the *rep* gene was placed downstream of the ITR cassette in the forward orientation (211). In addition, the development of conditional Rep expression systems has also been described to improve HSV/AAV hybrid vectors titers (118). Another strategy that could increase HSV/AAV hybrid vector titers and improve the rate of site-specific insertion of the ITR-flanked transgene cassette is the appropriate use of the AAV p5 promoter, adequately placing it into the transgene cassette (170).

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2. Experimental Work

The results section of this thesis is divided into 3 main parts: 2.1. HSV-1 compartment dynamics, which includes a manuscript published in the Journal of Virology entitled: *Live Visualization of Herpes Simplex Virus Type 1 Compartment Dynamics*; 2.2. Visualization of nuclear entry of HSV-1 genomes; and 2.3. Applications in gene/protein delivery, which includes a review article published in The Open Virology Journal, entitled: *Herpes Simplex Virus Type 1/Adeno-Associated Virus Hybrid Vectors*. Each part contains separate Aims, Materials and Methods, Results, Conclusions, and Reference sections.

2.1. HSV-1 compartment dynamics

2.1.1. Manuscript

Live Visualization of Herpes Simplex Virus Type 1 Compartment Dynamics

Anna Paula de Oliveira, Daniel L. Glauser, Andrea S. Laimbacher, Regina Strasser, Elisabeth M. Schraner, Peter Wild, Urs Ziegler, Xandra O. Breakefield, Mathias Ackermann, and Cornel Fraefel.

Contributions:

Construction of recombinant viruses: Anna Paula de Oliveira

Immunoprecipitation/Western Blot: Anna Paula de Oliveira, Andrea S. Laimbacher

Virus replication, purification, growth kinetics: Anna Paula de Oliveira, Regina Strasser

Immunofluorescence/Confocal microscopy: Anna Paula de Oliveira, Daniel L. Glauser

Fluorescence *in situ* hybridisation (FISH): Daniel L. Glauser

Electronmicroscopy: Anna Paula de Oliveira, Elisabeth M. Schraner, Peter Wild, Urs Ziegler

Manuscript writing, Figures: Anna Paula de Oliveira, Daniel L. Glauser, Cornel Fraefel, Mathias Ackermman

(Manuscript in the Appendix)

2.1.2. Conclusion

Recombinant viruses encoding specific viral proteins fused with autofluorescent proteins have been widely used for understanding the many aspects of viral life cycle, such as trafficking, assembly, and maturation (1, 3, 9, 10, 12). In the work presented in the manuscript, the construction of a triple fluorescent recombinant HSV-1 which encodes proteins from three different viral compartments, capsid, tegument, and envelope, fused to autofluorescent proteins, allowed studying interactions of different viral proteins simultaneously in live infected cells overtime. Increasing developments on microscopy techniques/equipments and image analysis algorithms have been enabling not only the visualization of subcellular structures and processes with higher fidelity, but also the assessment of informations at the single-molecule level (6, 8). In this sense, the combination of recombinant fluorescent HSV-1 with novel microscopy techniques would allow a better understanding of the dynamic interactions of autofluorescent recombinant HSV-1 proteins with the cellular environment.

For instance, experiments focusing on the interaction of triple fluorescent recombinant HSV-1 with the cellular transport machinery allied to high resolution-resonant scanning confocal microscopy and the tracking of single viral proteins (three-colour TIRF) (4), could shed more light onto the initial steps of entry and transport of incoming HSV-1 within the cytoplasm, or onto the comparison of viral composition during anterograde versus retrograde transport in axons. The combination of fluorescently labeled viruses with systems for live visualization of HSV-1 genomes, and super-resolution microscopy techniques (2, 7) would allow a more detailed assessment of the interactions among viral protein and viral genomes during replication and assembly processes.

The spatial organization of virion maturation, acquisition of tegument and envelope glycoproteins by capsids, could be investigated by integrating different microscopy techniques that permit identifying the presence and distribution of viral proteins within cellular compartments (e.g. dSTORM) (11) and their multiple interactions (e.g. ALEX with three-colour FRET) (5).

Although a challenging area, great advances in super-resolution microscopy techniques have been made, together with the development of brighter and photostable probes. These improvements would open a new dimension on visualizing and understanding the virus life cycle and its interactions with cellular processes within subcellular compartments or even at the single-molecule level.

2.1.3. References

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2.2. Visualization of nuclear entry of HSV-1 genomes

2.2.1. Aim

In order to visualize incoming HSV-1 genomes, a HSV-1 amplicon vector containing *lac* repressor (LacR) binding sites was packaged into HSV-1 particles that carry autofluorescent capsid proteins (VP26-mRFP) (Fig. 6). Delivery of the HSV-1 amplicon genome was expected to be detectable in infected cells constitutively expressing the LacR fused to the enhanced green fluorescent protein (EGFP) (Fig. 7).

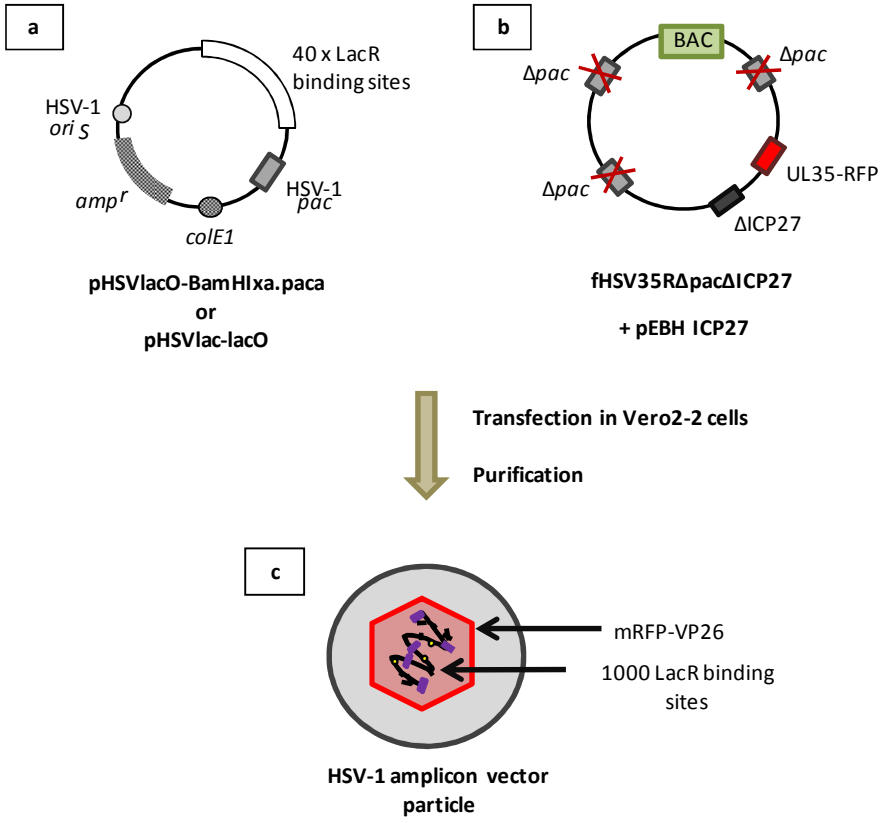

Figure 6. Production of the HSV-1 amplicon-35R/lacO vector. Vero2-2 cells were co-transfected with (a) an HSV-1 amplicon plasmid that carries 40 lac repressor (LacR) binding sites, contained in the lacO sequences, (b) a replication-competent packaging-defective HSV-1 helper BAC DNA that encodes the VP26 capsid protein fused to the monomeric red fluorescent protein (mRFP) (fHSV35RΔpacΔICP27), and the helper plasmid, pEBHICP27, that encodes the HSV-1 ICP27 protein. (c) Cells were harvested 3 days post-transfection and HSV-1 amplicon-35R/lacO vectors that contained around 1000 LacR binding sites per HSV-1 amplicon vector particle and the mRFP-VP26 fusion protein on the capsid were purified.

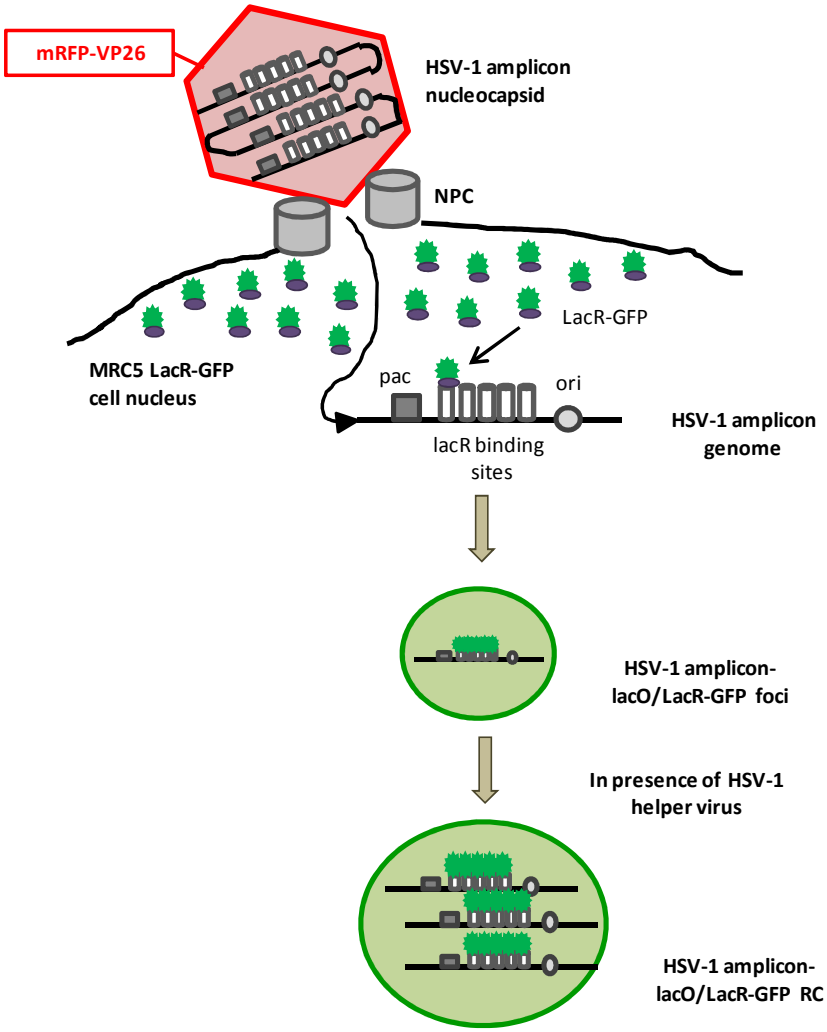

Figure 7. Strategy to visualize incoming HSV-1 amplicon-35R/lacO vector genomes into the cell nucleus. MRC5 LacR-GFP cells, constitutively expressing the lac repressor (LacR) fused to the green fluorescent protein (GFP), were infected with HSV-1 amplicon-35R/lacO vectors. Upon docking of the HSV-1 amplicon-35R/lacO vector nucleocapsid onto the nuclear pore complex, the HSV-1 amplicon-lacO genome is released inside the nucleus where LacR-GFP fusion protein can attach to approximately 1000 LacR binding sites per HSV-1 amplicon-35R/lacO genome. The accumulation of LacR-GFP binding to the HSV-1 amplicon-35R/lacO genome may support visualization of the incoming genomes as bright green fluorescent foci inside the nucleus. Incoming vector genomes may develop into replication compartments (RC) in presence of a HSV-1 helper virus.

2.2.2. Material and Methods

Experiments were performed as described in the manuscript above (5), except for some modifications that are specified here.

Cells

VERO 2-2 cells (8), HeLa LacR-GFP cells, and MRC5 LacR-GFP cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 100 units/ml penicillin G, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B. Specific antibiotic requirements for cellular selection were included in addition as follows: 500 µg/ml G418 for Vero 2-2 cells, 1 µg/ml puromycin for HeLa LacR-GFP cells and 100 µg/ml hygromycin for MRC5 LacR-GFP cells. HeLa LacR-GFP and MRC5 LacR-GFP cells both stably express the *lac* repressor, LacR, fused to green fluorescent protein, GFP (1).

Viruses

The following viruses were used: (i) wild type HSV-1 strain F, and (ii) recombinant vECFP-ICP4 expressing the enhanced cyan fluorescent protein (ECFP) fused to the N-terminus of ICP4 under the native IE3 promoter (9), kindly provided by R.D. Everett (MRC Virology Unit Glasgow, UK).

Plasmids

The pHSVlacO-*Bam*HIxa.paca DNA is a HSV-1 amplicon plasmid containing 5 *lac* operator (lacO) sequences, that comprise 40 LacR binding sites, and the coding sequence of US1, US2, and US3 genes involved in the transcriptional machinery of HSV-1 (Fraefel, unpublished). pHSVlac-lacO is a HSV-1 amplicon plasmid containing lacO sequences and the E. *coli lacZ* reporter gene under transcriptional control of the HSV-1 IE 4/5 promoter (Fraefel, unpublished).

Bacterial artificial chromosomes fHSV35RΔpacΔICP27 or fHSVΔpacΔICP27, and plasmid pEBHICP27 together represent a replication-competent, packaging defective HSV-1 genome (6) and were used to provide HSV-1 helper functions for packaging of amplicon plasmids into HSV-1 particles.

Production of HSV-1 amplicon vector stocks

HSV-1 amplicons were produced as previously described (3, 6) and outlined here. i) Packaging into HSV-1 virions. Vero2-2 or HeLa LacR-GFP cells seeded at 1.2 x 10^6 cells per 6 cm-diameter plate were transfected using Lipofectamine Plus Reagent according to manufacturer's instructions (Invitrogen). Purified amplicon plasmids used in this work included pHSVlac-lacO or pHSVlacO-*Bam*HIxa.paca (0.5 µg). Helper functions were provided by co-transfection with either fHSV35RΔpacΔICP27 or fHSVΔpacΔICP27 (2 µg) and pEBHICP27 (0.1 µg). Cells were harvested 3 days post-transfection, frozen and thawed 3 times, and sonicated at 20 % for 20 s on ice. Supernatant was clarified by centrifugation at 3'500 rpm for 10 min at 4 °C. ii) Purification of HSV-1 amplicon vector stocks. After the clarification step, the viral supernatant was added on top of a 25 % sucrose (in HBSS) cushion in a Beckman Ultra-Clear 25 x 89-mm centrifuge tube and centrifuged at 20'000 rpm in a Beckman AH629 rotor for 3 h at 16 °C. The supernatant was removed and the pellet was allowed to dissolve in HBSS at 4 °C overnight. Aliquots were stored at – 80 °C.

Titration of amplicon vector stocks

i) Infection of Vero2-2 cells with HSV-1 amplicon vectors. Vero2-2 cells were seeded at 10^5 cells per well in a 24 well-plate. The next day, cells were washed with PBS and incubated with serial dilutions (1:5, 1:25, 1:250) of the HSV-1 amplicon vector stocks for 1 h in DMEM. Medium was changed to DMEM supplemented with 2 % FBS, and cells were incubated for 3 days at 37 °C and 5 % CO_2. ii) X-Gal staining. Three days after infection with HSV-1 amplicon vector pHSVlac-lacO, which expresses the E. *coli lacZ* reporter gene, cells were washed with PBS , fixed with 4 % paraformaldehyde for 20 min at room temperature (RT), and incubated for 16 h with 1 mg/ml X-Gal (5 -Bromo-4-chloro-3-indolyl-beta-D-galactopyranoside in dimethylformamide) in staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl, 0.01 % sodium deoxycholate, 0.02 % Nonidet P-40 (NP-40) in PBS) at 37 °C and 5 % CO_2. Titers were determined by counting X-Gal positive cells using an inverted light microscope.

Electron microscopy

(i) Negative Staining. Purified virus samples were adsorbed to carbon coated parlodion films mounted on 300 mesh/inch copper grids (EMS, Fort Washington, PA, USA) for 10 min at RT, washed once with H_2O, and stained with 2 % phosphotungstenic acid pH7.0 (Aldrich, Steinheim, Germany) for 1 min. Specimens were analyzed in a transmission electron microscope (CM12, Philips, Eindhoven,

The Netherlands) equipped with a charge-coupled device (CCD) camera (Ultrascan 1000, Gatan, Pleasanton, CA, USA) at an acceleration voltage of 100 kV.

Immunofluorescence analysis

Immunofluorescence analysis was performed as described previously (5). For staining ICP8, a mouse anti-HSV-1 ICP8 MAb (7381; 1:500; kindly provided by R.D. Everett, MRC Virology Unit, Glasgow, UK) and a goat anti-mouse IgG (H+L)-AF594 (1:200, Molecular Probes, Invitrogen, Basel, Switzerland) were used.

Confocal laser scanning microscopy

Confocal laser scanning microscopy *(CLSM)* was performed using a Leica TCS SP2 AOBS confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) with settings as previously described (5). Cells were also visualized using a Leica Resonant, APD, DMI6000B SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany), equipped with an incubation chamber (THE BOX; Live Imaging Services, Reinach, Switzerland), a temperature-control device (THE CUBE; Live Imaging Services, Reinach, Switzerland), and a gas mixer (THE BRICK; Live Imaging Services, Reinach, Switzerland). Settings for the SP5 microscope were as follows: EGFP, excitation at 488 nm and recording at 500-543 nm; mRFP, excitation at 594 nm, and recording at 609-700 nm.

2.2.3. Results

Production of HSV-1 amplicon vectors containing arrays of LacR binding sites

In order to visualize incoming HSV-1 genomes, HSV-1 amplicon vectors were produced that combine the helper-free amplicon packaging system and the EGFP-LacR-lacO detection method (6, 9, 10). For packaging of the amplicon DNA into HSV-1 particles, Vero2-2 cells were co-transfected with the amplicon plasmid pHSVlacO-*Bam*HIxa.paca and the replication-competent packaging-defective HSV-1 helper DNA, fHSV35RΔpacΔICP27 (Fig. 6). Amplicon plasmids contain the minimal cis-acting elements for HSV-1 replication: the origin of viral DNA replication (*ori*), and the packaging signal (*pac*). In addition, pHSVlacO-*Bam*HIxa.paca contains 40 copies of LacR binding sites (Fig. 6a). The HSV-1 helper functions provided by fHSV35RΔpacΔICP27 and pEBHICP27 supported the replication of the amplicon plasmid to form concatemeric molecules, thus amplifying the number of LacR-GFP binding sites (Fig. 6b). In addition, it also provided the structural proteins necessary for the packaging of the amplicon genome into HSV-1 particles containing the VP26 capsid protein fused to the monomeric red fluorescent protein (VP26-mRFP, or 35R), resulting in the HSV-1 amplicon-35R/lacO vector (Fig. 6c). As pHSVlacO-*Bam*HIxa.paca does not contain a reporter gene, titration of the vector stock was not possible. Therefore, an additional HSV-1 amplicon vector stock was produced using the amplicon plasmid pHSVlac-lacO as the template for replication and packaging into HSV-1 particles. pHSVlac-lacO contains in addition to the 40 copies of LacR binding sites, the E. *coli lac*Z gene which allows titration of vector stocks by X-Gal staining. Titers obtained in Vero cells ranged from 10^6 TU/ml before concentration to around 10^7 TU/ml after concentration by ultracentrifugation.

Structural characterization of HSV-1 amplicon-35R/lacO vector particles

The formation of HSV-1 particles derived from HSV-1 amplicon packaging was analyzed by electronmicroscopy. Purified HSV-1 amplicon-35R/lacO vector particles contained all HSV-1 viral structures: envelope, tegument, and capsid (Fig. 8A); unenveloped HSV-1 amplicon vector particles were also observed (Fig. 8B).

Visualization of incoming HSV-1 amplicon genomes

The visualization of the delivery of HSV-1 amplicon genomes into the host cell nucleus was based on the LacR-GFP/lacO detection method. In this method, LacR-GFP fusion protein can bind to the LacR binding sites presented by the lacO sequences. Each lacO sequence contains approximately 8 LacR

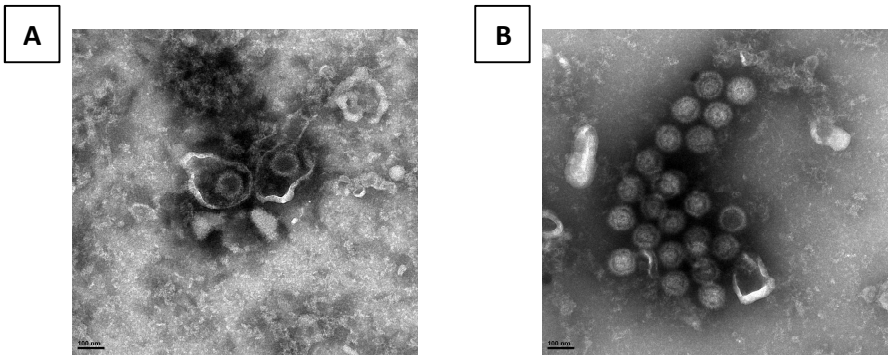

Figure 8. HSV-1 amplicon-35R/lacO vector particles. HSV-1 amplicon-35R/lacO vectors were purified through a 25 % sucrose gradient and prepared for negative staining electron microscopy. (A) Complete HSV-1 amplicon-35R/lacO vector particles consisting of capsid, tegument, and envelope. (B) Unenveloped HSV-1 amplicon-35R/lacO vector particles.

binding sites. Since the amplicon contained 5 lacO sequences, a single vector genome would have approximately 1000 LacR binding sites. MRC5 LacR-GFP cells, constitutively expressing the LacR-GFP fusion protein, were infected with HSV-1 amplicon vectors containing concatemers of LacR binding sites in its genome and the VP26-capsid protein fused to mRFP. Upon docking of HSV-1 amplicon nucleocapsids at the nuclear pore complex (NPC) the HSV-1 amplicon genome is released into the cell nucleus where the resident LacR-GFP proteins may attach to the LacR binding sites present at the HSV-1 amplicon genomes, thereby allowing visualization (Fig. 7).

Mock-infected MRC5 LacR-GFP cells showed a characteristic diffuse GFP background, related to the constitutive expression of the fusion gene (Figure 9 A). Early in infection, incoming HSV-1 amplicon vector particles were identified as red dots (VP26-mRFP) in the cytoplasm approaching the nucleus during live cell imaging. Inside the nucleus, individual bright LacR-GFP/lacO dots were observed as early as 2 h p.i.. Juxtaposed VP26-mRFP and LacR-GFP/lacO dots were observed at the nuclear rim, possibly representing an amplicon genome released from the nucleocapsid into the nucleus (Fig. 9 B, insets). However, attempts to monitor the exact moment of HSV-1 amplicon genome delivery into the nucleus by live time lapse microscopy were not successful. Upon co-infection with vECFP-ICP4 at an MOI of 5, the LacR-GFP/lacO foci were found to colocalize with ICP4-ECFP, and HSV-1 ICP8 protein was observed juxtaposed to the LacR-GFP/lacO foci (Fig. 10, 4 h p.i.). Later in infection, the LacR-GFP/lacO foci were demonstrated to develop into replication compartments (RC) (Fig. 10, 8 h p.i.), indicating that the amplicon vectors delivered replication competent vector genomes.

Another strategy tested to visualize incoming amplicon genomes was to package the amplicon plasmid pHSVlacO-*Bam*HIxa.paca in the presence of LacR-GFP, for instance, in HeLa LacR-GFP cells, resulting in the HSV-1 amplicon-35R-lacO/LacR-GFP vector. This was expected to allow the packaging

of the LacR-GFP/lacO complex within the HSV-1 amplicon vector particle and to facilitate the visualization of incoming HSV-1 amplicon genomes in any cell line avoiding the GFP background present in the nucleus of cells that constitutively express the LacR-GFP fusion protein. Although upon infection of Vero cells some VP26-mRFP and LacR-GFP/lacO dots were observed to colocalize in the cytoplasm, it was not possible to visualize the LacR-GFP/lacO foci inside the nucleus (Fig. 11). This could be interpreted as an impediment of the LacR-GFP/lacO structure in crossing the nuclear pore and entering the nucleus.

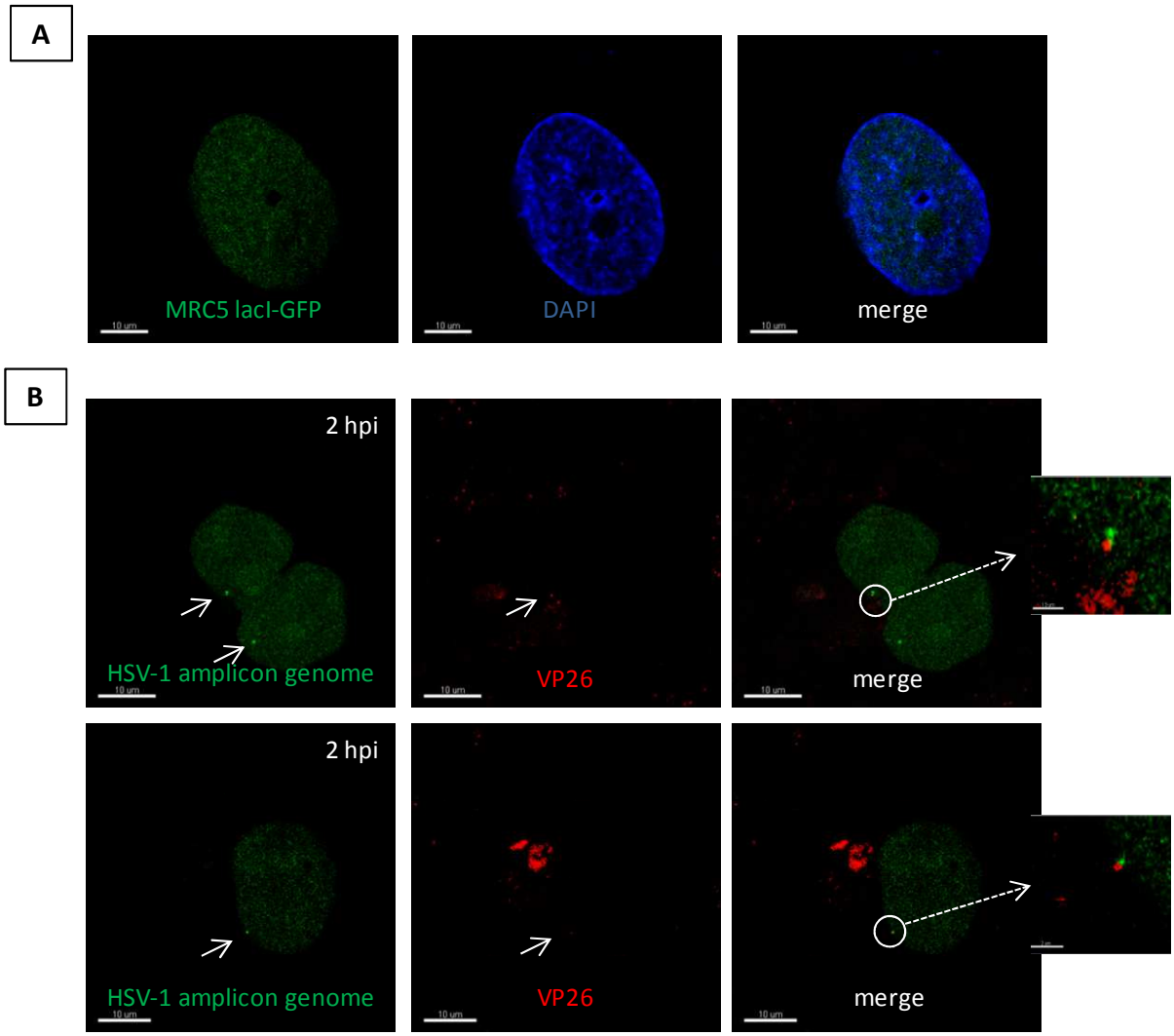

Figure 9. Visualization of incoming HSV-1 amplicon-35R/lacO vector genomes. MRC5 LacR-GFP cells were (A) mock-infected or (B) infected with HSV-1 amplicon-35R/lacO vectors. Cells were fixed at 1 and 2 h p.i., and visualized by CLSM (SP5-Ressonant mode) with settings specific for mRFP (mRFP-VP26 fusion protein), GFP (LacR-GFP/lacO foci), and DAPI. Insets show a high magnification of the circled areas. Arrows point to juxtapositions of mRFP-VP26 and LacR-GFP/lacO foci. Scale bar = 10 µm.

2.2.4. Discussion and Conclusions

The use of the HSV-1 amplicon vector system combined with the LacR-GFP/lacO detection system has previously been described and used to visualize the formation of HSV-1 replication compartments (2, 9). In the present study, an HSV-1 amplicon vector containing the lacO sequences and the capsid VP26 protein fused to the monomeric red fluorescent protein (mRFP) was used for tracking the viral nucleocapsid in the infected cell.

Live imaging showed many capsids approaching the nuclear rim but did not allow detection of the exact moment of HSV-1 amplicon genome delivery into the nucleus. The analysis of fixed infected cells however revealed juxtaposed LacR-GFP/lacO and VP26-mRFP foci at the nuclear rim (Fig. 9B, insets) which might be interpreted as HSV-1 amplicon genomes released from the nucleocapsid into the nucleus. Of note, the LacR-GFP/lacO foci found inside the nucleus were demonstrated to be incoming HSV-1 amplicon genomes as they were shown to associate with the HSV-1 ICP4 protein early during co-infection. These LacR-GFP/lacO foci were also shown to be juxtaposed to ICP8 foci (Fig. 10, 4 h p.i.), and to develop into RCs later in infection in the presence of helper functions provided by vECFP-ICP4 (Fig. 10, 8 h p.i.).

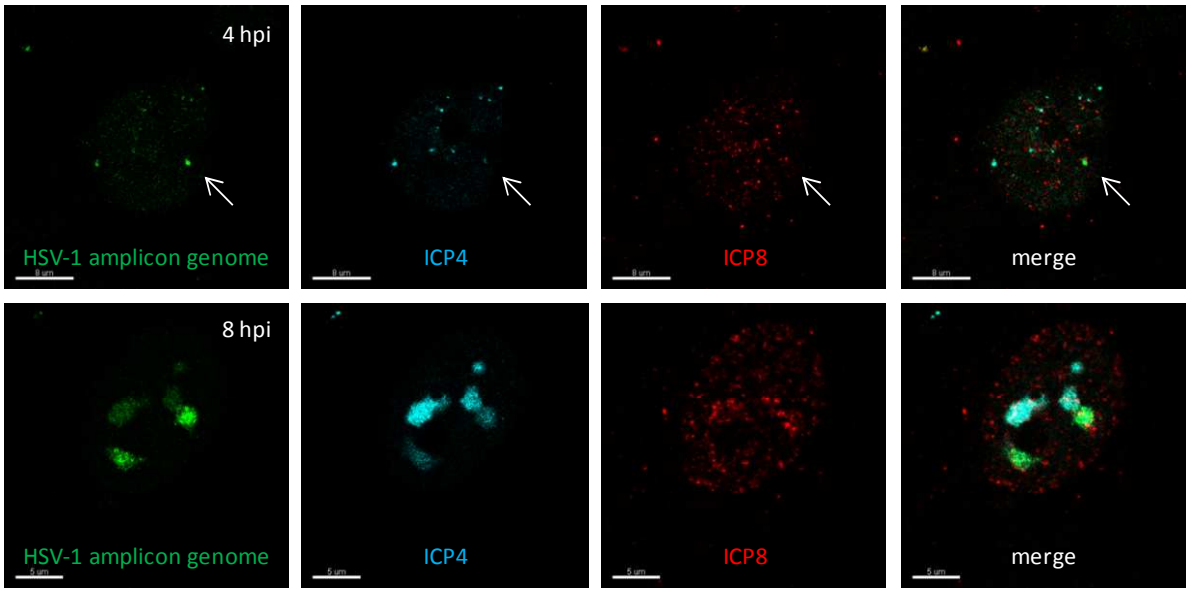

Figure 10. Incoming HSV-1 amplicon-lacO genomes, interactions with HSV-1 proteins and formation of replication compartments. MRC5 LacR-GFP cells were co-infected with vECFP-ICP4 and the HSV-1 amplicon-lacO vector, fixed at 4 or 8 h p.i. and stained with antibodies specific for ICP8. Cells were visualized by CLSM (SP2) with settings specific for GFP (LacR-GFP/lacO foci), ECFP (ECFP-ICP4 fusion protein), and AF594 (ICP8 or ICP27). Arrows point to colocalizations between GFP, CFP, and RFP. Scale bar = 10 µm.

Investigations on HSV-1 genome delivery using atomic force microscopy have described the HSV-1 genome as a highly condensed, rod-like structure entering the nucleus through the widening of the NPC central channel (7). However, that study also did not detect the exact moment of genome release from the capsids (7). The translocation of the HSV-1 genome into the cell nucleus has been described as a slow process, in which the viral genome remains associated with the cytoplasmic side of the NPC, while the capsids rapidly dissociate from the NPC and disassemble after release of the viral DNA (4, 7). According to these observations, it seems that visualizing the right moment of the nucleocapsid docking at the NPC followed by the genome release into the nucleus is indeed very challenging, as it seems to be a very transient event. The association of super-resolution and fast-tracking microscopy would be of great value for the observation of these events.

A HSV-1 amplicon harboring the LacR-GFP/lacO complex (HSV-1 amplicon-35R-lacO/LacR-GFP) was also used in order to avoid the GFP background present in the nucleus of cells that constitutively express the LacR-GFP fusion protein. Although it appeared to be possible to package the LacR-GFP bound to the lacR binding sites on the HSV-1 amplicon genome, as mRFP-VP26 dots were visualized colocalizing with LacR-GFP/lacO dots in the cytoplasm and approaching the cell nucleus (Fig. 11), no LacR-GFP/lacO foci were observed inside the nucleus. One explanation for this could be that the complex formed on the HSV-1 amplicon genome prevents it from entering the NPC.

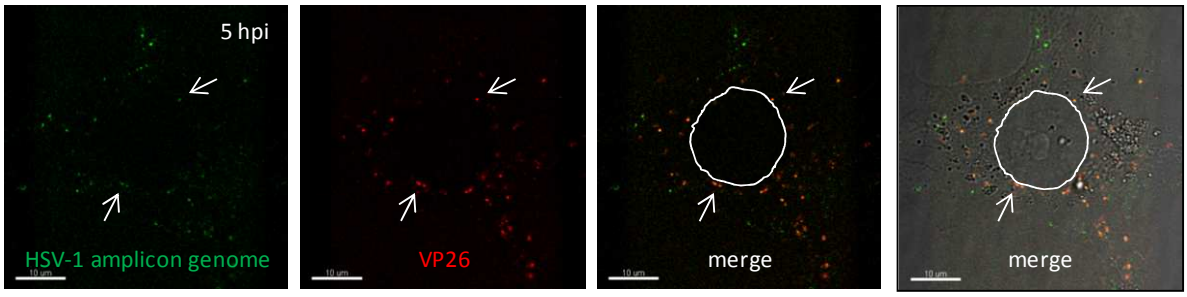

Figure 11. Incoming HSV-1 amplicon-35R-lacO/LacRGFP vector. HSV-1 vectors containing the pHSVlacO-*Bam*HIxa.paca amplicon genome were produced in HeLa LacR-GFP cells expressing the LacR-EGFP fusion protein and using fHSV35RΔpacΔICP27 and pEBHICP27 to provide helper functions. The resulting vector particles were expected to have LacR-GFP fusion protein bound to lacO sequences inside red fluorescent capsids. Vero cells infected with these vectors were fixed at 5 h p.i. and visualized by CLSM (SP5-Ressonant mode) with settings specific for mRFP (mRFP-VP26 fusion protein), and GFP (LacR-GFP/lacO foci). Arrows point to colocalizations between GFP and mRFP. The nuclear rim is depicted on merge images. Scale bar = 10 µm.

2.2.5. References

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2.3. Applications in gene/protein delivery

The biological properties of HSV-1, such as its large genome, a broad cell tropism and the ability to establish latency have been exploited for the development of vectors for gene therapy. While HSV-1 based vectors are promising gene delivery vehicles with many advantageous properties, they also have the major deficit that transgene expression is transient. One strategy to overcome this deficit has been the combination of genetic elements from HSV-1 with those from AAV2 in a single vector. In particular, the AAV2 *rep* genes and the AAV2 inverted terminal repeats (ITRs) are sufficient to mediate the integration of the AAV2 genome into a specific locus, termed AAVS1, on human chromosome 19 (2). HSV/AAV hybrid amplicon vectors have indeed been demonstrated to (i) facilitate the genomic integration of transgene sequences into AAVS1 and (ii) support long-term transgene expression. This third part of the thesis includes a Review Article by de Oliveira and Fraefel (4) on HSV/AAV hybrid vectors that serves as an introduction (2.3.1.), and the description of two novel HSV/AAV hybrid vector applications/strategies: (2.3.2.) the functional delivery of AAV2 Rep protein by HSV-1 vector particles and (2.3.3.) the use of HSV-1 vectors to launch the production of rAAV2 vectors in the HSV-1 vector infected cell.

2.3.1. Manuscript

Herpes Simplex Virus Type 1/Adeno-Associated Virus Hybrid Vectors

Anna Paula de Oliveira and Cornel Fraefel

The Open Virology Journal, 2010, 4, 109-122

Contributions:

Manuscript writing and figures: Anna Paula de Oliveira and Cornel Fraefel

(Manuscript in the Appendix)

2.3.2. Functional delivery of AAV Rep protein by recombinant HSV-1 vector particles

2.3.2.1. Aims

The possibility to deliver one or multiple functional heterologous proteins fused to structural HSV-1 proteins in the virion was demonstrated by the construction of recombinant HSV-1 that incorporated autofluorescent proteins fused simultaneously to three viral proteins from different viral compartments (3). A possible application of this strategy was explored by the packaging of HSV/AAV hybrid amplicon vectors into HSV-1 particles that contain the AAV2 Rep protein fused with the VP16 tegument protein. The fusion of AAV2 Rep with VP16 would allow for its delivery into the cell nucleus of the vector infected-cell and potentially for mediating the integration of the AAV2 ITR-flanked transgene cassette delivered by the vector genome (Fig. 12).

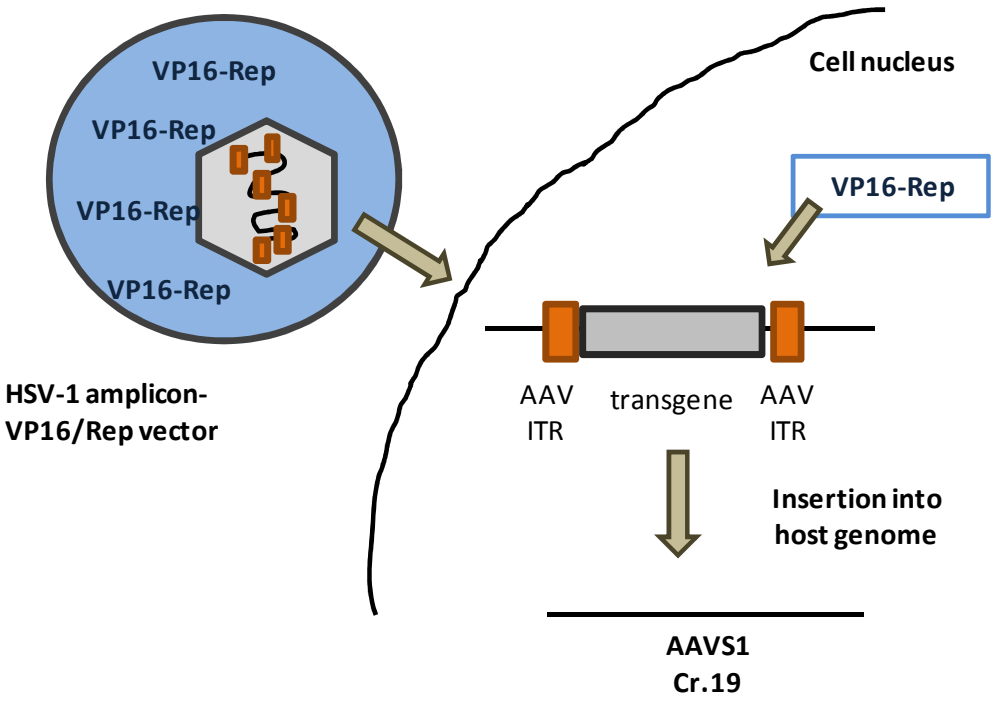

Figure 12. Delivery of AAV Rep protein fused to the HSV-1 VP16 tegument protein by a HSV/AAV hybrid amplicon vector. A HSV/AAV hybrid amplicon plasmid containing a transgene cassette flanked by the AAV ITRs is packaged into HSV-1 particles by using a bacterial artificial chromosome that contains the HSV-1 genome with deletions in the ICP27 gene and the *pac* signals, and the UL48 (VP16 tegument protein) gene fused with the AAV *rep*68/78 gene. Upon infection of cells, the HSV/AAV amplicon genome is released inside the cell nucleus where the Rep-VP16 fusion tegument protein initiates the integration of the ITR-flanked transgene cassette into the AAVS1 site of the human chromosome 19.

2.3.2.2. Material and Methods

Cells

Vero2-2 (5) and Vero cells were maintained in Dulbecco's modified Eagle medium supplemented with 10 % fetal bovine serum, 100 units/ml penicillin G, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B. For Vero2-2 cells, the medium was supplemented with 500 µg/ml G418.

Plasmids

Plasmids pAAVlacO contains 40 LacR binding sites flanked by the AAV ITRs, and pSV2-EYFP-LacR encodes the LacR fused to the enhanced yellow fluorescent protein (EYFP) (1, 2, 7). pRep expresses all four *rep* genes (*rep*78/68/52/40) from the AAV p5 and p19 promoters (1, 2).

Construction of a recombinant HSV-1 expressing a VP16-Rep fusion protein

A recombinant HSV-1 expressing the AAV2 Rep protein fused to the HSV-1 VP16-tegument protein (rHSV48Rep) was constructed by homologous recombination in E. *coli* SW102 and *galK* selection/counterselection (Fraefel, unpublished data) (8) using a bacterial artificial chromosome (BAC) cloned HSV-1 strain F genome (6).

Western Analysis

The expression of the AAV2 Rep protein fused to the HSV-1 VP16-tegument protein was confirmed by Western analysis. The procedures were as described previously with the exception that the first antibodies used here were specific for AAV Rep (1:200 in PBS-T20; cat n° 10R-A111A, clone 303.9; Fitzgerald Industries International Inc., Concord, MA, USA) or HSV-1 VP16 (1:500 d in PBS-T20; MAb LP1).

Visualization of AAV replication Compartments

Vero2-2 cells seeded onto coverslips (17 mm-thick, n° 1) in 24 well plates at 10^5 cells per well were transfected using Lipofectamine Plus Reagent as described by the manufacturer (Invitrogen). The amounts of plasmids used were as follows: pAAVlacO (0.05 g/l), pSV2-EYFP-LacR (0.01 g/l), pBs (0.05 g/l), pRep (0.01 g/l). Helper functions were provided by superinfection with wtHSV-1 or rHSV-48Rep at an MOI of 5 at 5 h post-transfection. Cells were fixed 16 h after transfection, and examined by

CLSM confocal fluorescence microscopy. Confocal microscopy images were processed with Imaris 4.1.1 (Bitplane AG, zurich, Switzerland).

2.3.2.3. Results

The recombinant HSV-1 virus encoding the HSV-1 VP16-tegument protein fused to the AAV2 Rep protein (rHSV48-Rep) was constructed via homologous recombination in E. *coli* using a BAC-cloned HSV-1 strain F genome (6) and the *galK* positive/negative selection method (8) as described previously (3) and in Materials and Methods. The recombinant rHSV48-Rep virus was rescued through the excision of the BAC sequences from the recombinant HSV-1 genome mediated by co-transfection with Cre recombinase-expressing plasmid, p116. The progeny virus was harvested and plaque-purified. Recombinant HSV48-Rep reached titers of 1.23 x 10^7 PFU/ml to 2.18 x 10^8 PFU/ml after concentration. In order to verify the expression of the VP16-Rep fusion protein, Vero cells were mock-infected or infected with either wtHSV-1, rHSV48-Rep, or co-infected with wt HSV-1 and wtAAV2, and harvested after reaching a cytopathic effect (CPE) of 90 %. Western blotting of lysates of cells infected with either the wtHSV-1, or by co-infection with wtHSV-1 and wtAAV2, the native HSV-1 VP16 and the AAV2 Rep proteins were detected using an antibody specific for VP16 (Fig. 13A) or an antibody specific for Rep (Fig. 13B), respectively. In cells infected with the rHSV48-Rep the native VP16 as well as the native Rep protein were replaced by a band higher than 120 kDa detected with both antibodies, anti-VP16 (Fig. 13A) and anti-Rep (Fig. 13B).

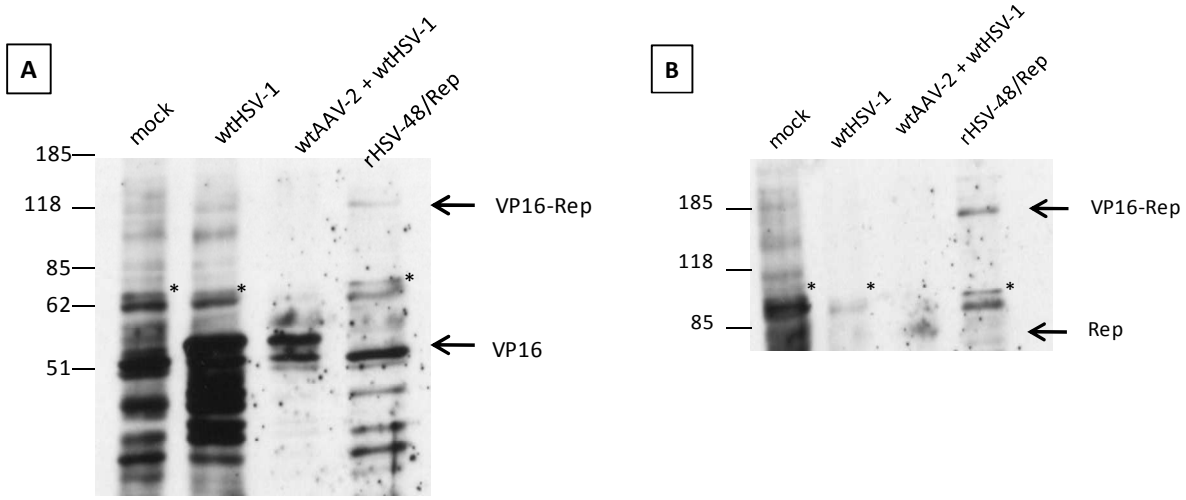

Figure 13. Expression of the VP16-Rep fusion protein in infected cells. Vero cells were mock infected or infected with either wtHSV-1, or rHSV48-Rep, or co-infected with wtAAV2 and wtHSV-1. The cells were harvested when the CPE reached 90 % and analyzed by Western blotting with antibodies specific for (A) VP16 or (B) Rep proteins. Sizes of molecular weight standards are indicated. Unspecific bands marked with a star (*).

The ability of the Rep protein, when fused with VP16, to induce the replication of an ITR flanked-transgene cassette was evaluated by the visualization of AAV replication compartments using lacO-LacR interaction combined with autofluorescent proteins (1). For this, Vero2-2 cells were co-transfected with a plasmid carrying lacO sequences flanked by the AAV ITRs (pAAV-lacO) and a plasmid encoding the LacR fused to the EYFP (pSV2-EYFP-LacR) (Fig. 14). For some transfection experiments a *rep* expressing plasmid, pRep, was included (Fig. 14a). To provide helper virus functions for the replication of the AAV ITRs flanked transgene cassette, the transfected cells were infected with wt HSV-1 (Fig. 14a, and b) or rHSV-48-Rep (Fig. 14c). In the presence of Rep (pRep) and wtHSV-1 helpervirus, AAV replication compartments (RC) were formed in the host cell nucleus (Fig. 14a). In the absence of pRep, no AAV RCs were formed (Fig. 14b). When rHSV-48Rep was used as the helpervirus, AAV RCs were formed also in the absence of pRep, indicating that the Rep-VP16 fusion protein was functional in supporting the replication of AAV genomes (Fig. 14c).

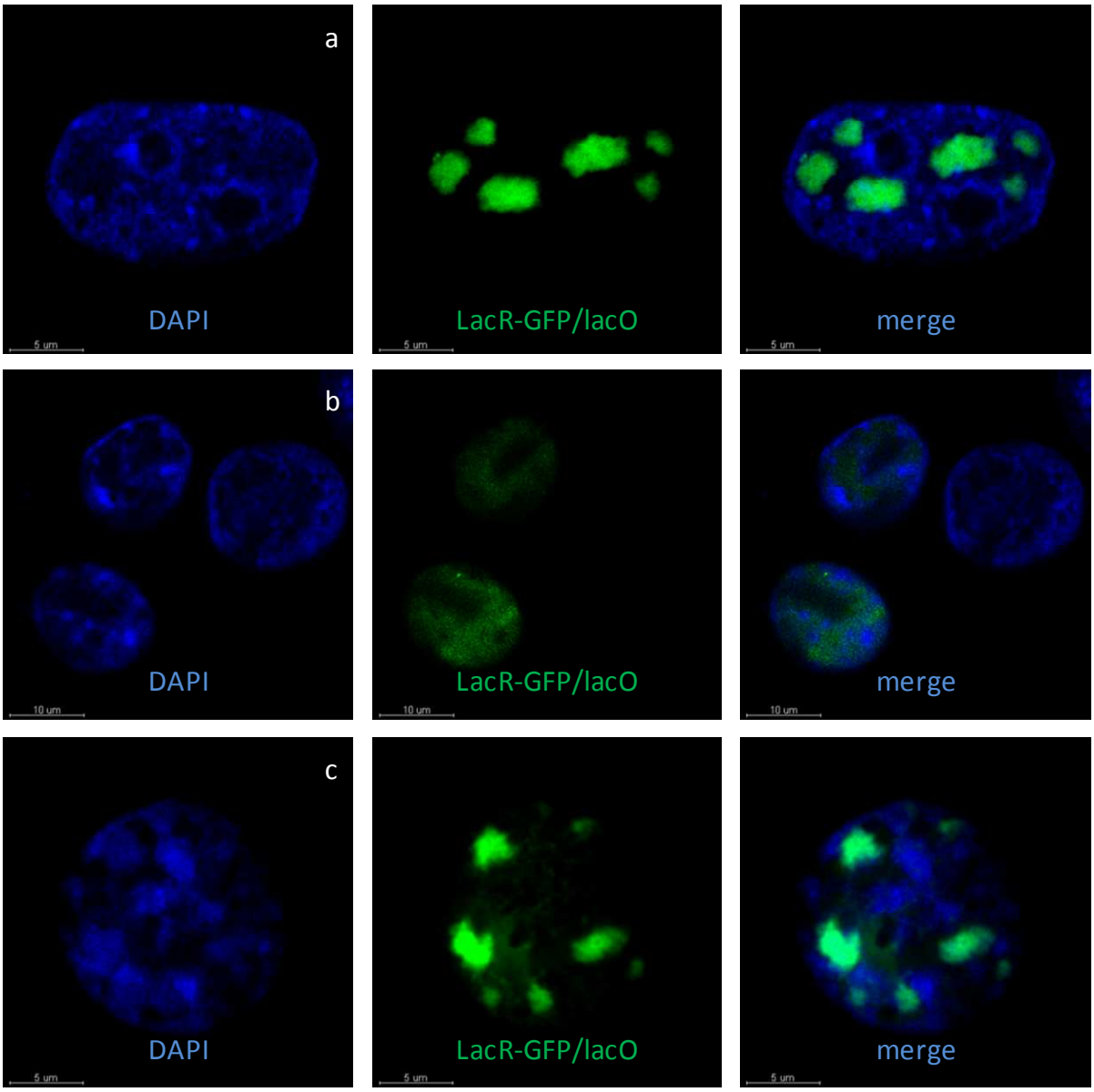

Figure 14. Visualization of rAAV-lacO DNA replication in Vero2-2 cells. Vero2-2 cells were co-transfected with pAAV-lacO, pSV2-EYFP-LacR and infected either with (a, b) wtHSV-1 or with (c) rHSV48-Rep. In (a) a *rep* expressing plasmid, pRep was included. Confocal laser scanning microscopy was performed with settings for EYFP (AAV replication compartments), and DAPI.

2.3.2.4. Discussion and Conclusions

The construction of a recombinant HSV-1 in which the tegument protein VP16 was fused to the AAV2 Rep protein (rHSV-48Rep) was demonstrated. The AAV2 Rep protein was shown to maintain its function when fused to VP16, as it supported the formation of AAV2 RCs. A next step would include the packaging of an HSV/AAV hybrid vector that contains an AAV2 ITR flanked transgene cassette into HSV-1 virions harboring the Rep-VP16 fusion in the tegument. This would require the deletion of the DNA cleavage/packaging signals from the BAC cloned rHSV-48Rep and use of the resulting replication-competent, packaging-defective HSV-1 genome to provide helper functions for the replication and packaging of the hybrid amplicon vector. In addition, it would be necessary to assess whether the Rep-VP16 fusion protein would mediate the site-specific integration of the AAV ITR-flanked transgene cassette into AAVS1 on hybrid vector transduced cells. The delivery of AAV Rep as a fusion protein within the vector particle to mediate the site-specific integration of an AAV ITR flanked transgene delivered by the vector genome, as opposed to the delivery of the *rep* gene encoded from the vector genome, would have several advantages, including: (i) fusion of *rep* to the late gene UL48 (VP16) would avoid inhibition of the HSV-1 genome replication at early phases; (ii) *rep* would not be integrated into the host cell genome; (iii) Rep protein would still be functional and able to mediate the integration of the ITR cassette flanking the transgene of interest into the AAVS1 site of the host cell chromosome 19.

2.3.2.5. References

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2.3.3. Recombinant HSV-1 vectors that launch the production of recombinant AAV vectors in infected cells.

2.3.3.1. Aims

The second HSV/AAV hybrid vector strategy/application is based on the idea that a recombinant HSV-1 encoding AAV2 Rep and Cap and containing an AAV2 ITR flanked transgene cassette would support the launch of rAAV2 vectors in the rHSV-1 vector infected cell (Fig. 15). For this, an oncolytic rHSV-1 vector (MGH1), encoding the AAV2 elements, and a fluorescent protein marker flanked by the AAV2 ITRs was constructed.

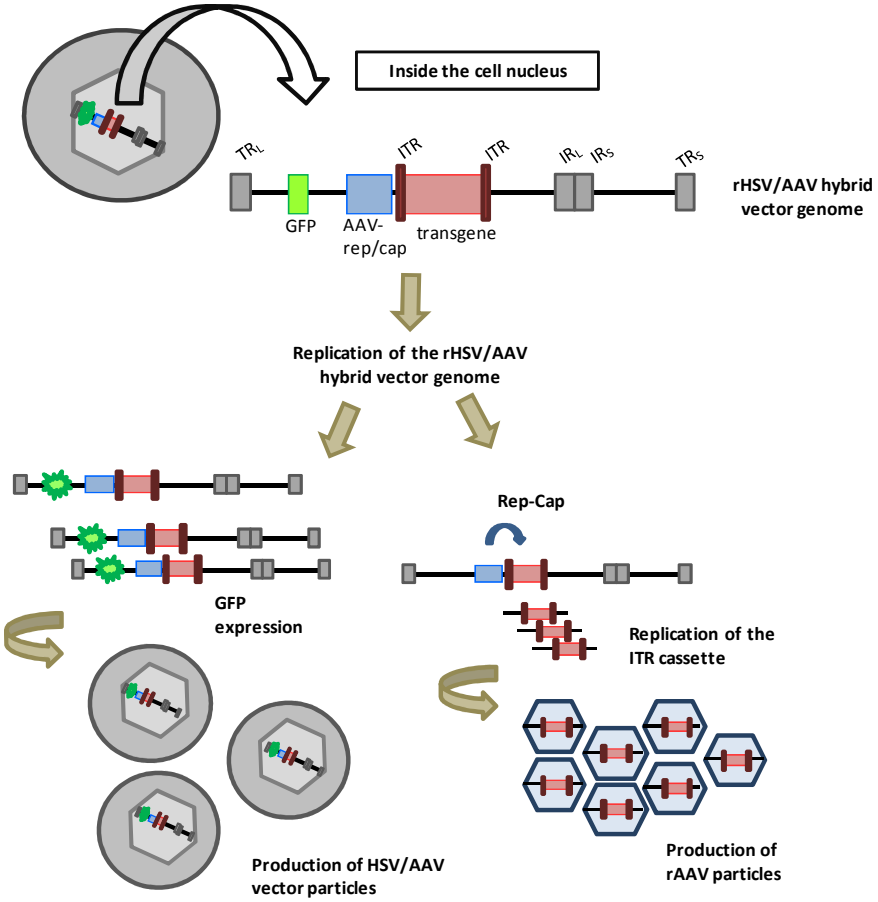

Figure 15. Schematic model of a recombinant HSV-1 vector that launches the production of rAAV vectors in infected cells. A rHSV/AAV hybrid vector was constructed that contained the AAV2 *rep* and *cap* genes, and a fluorescent protein marker flanked by the AAV2 ITRs. Upon infection, the rHSV/AAV genome replicates in the cell nucleus and forms progeny rHSV/AAV hybrid vector particles; it also expresses the AAV2 *rep* and *cap* genes. The AAV2 Cap proteins facilitate the formation of rAAV capsids. The Rep proteins together with HSV-1 helper functions induce the replication of the ITR transgene cassette. Single stranded ITR flanked transgene cassettes are packaged into rAAV particles that are released by the infected cell.

2.3.3.2. Material and Methods

Plasmids

i) Construction of the ITR cassette. First, plasmid CSCW-Gluc-ICFP (13) was digested with *Nhe* I and *Xho* I, and the small fragment of 564 bp corresponding to the hGluc sequence was separated by agarose gel electrophoresis and purified using the QiaEx Agarose Gel Extraction Kit (Quiagen, Invitrogen). The hGluc sequence was directly ligated (T4 DNA Ligase, New England Biolabs) into the CSCW2-IRES mCherry plasmid (13), previously digested with the same restriction enzymes (*Nhe* I and *Xho* I). The resulting plasmid containing both the hGluc and mCherry sequences was termed CSCW2-IRES-mCherry-Gluc. The pITRmpA plasmid contains the AAV2 ITR sequences flanking a multiple cloning site (MCS), and the bovine growth hormone poly A (bGhpA) signal (Fraefel et al., unpublished). The pITRmpA was linearized with *Sca* I restriction enzyme, made blunt, and dephosphorylated with calf intestinal phosphatase (CIP). The CSCW2-IRES-mCherry-Gluc plasmid was digested with *EcoR* I, and the 3.7 kbp fragment of interest was separated by agarose gel electrophoresis, purified (QiaEx Agarose Gel Extraction) and blunt end ligated into the MCS of the pITRmpA plasmid. The resulting plasmid, pITR-Gluc-mCherry, was confirmed by sequence analysis. The pITR-Gluc-mCherry plasmid was digested with *Bgl* II and *Bst*1107 I, and the 4.1 kbp fragment corresponding to the ITR cassette was purified (QiaEx Agarose Gel Extraction) for further cloning into the pTransfer plasmid. ii) Cloning of essential AAV components. The AAV2 *rep* and *cap* genes together with the native promoters were amplified by PCR using the following primers: p5 forward primer (5'-GGGATATCCGTGAATTACGTCATA GGGT-3') containing an *EcoR* V restriction site (underlined), and the reverse Cap primer (5'-GAAGATCTAAACTAGATAAGAAAGAAAT-3') containing a *Bgl* II restriction site (underlined). The pAV2 plasmid (7) containing a wild type AAV2 genome was used as template for the PCR. The PCR product was digested with *EcoR* V and *Bgl* II restriction enzymes and purified (QiaEx Agarose Gel Extraction). iii) Construction of the transfer vector. Plasmid pTransfer, a replication conditional plasmid containing a R6Kγ-ori (14), was digested with *Sma* I, columm purified, and then digested with *Bam* HI (Fig. 16A). The linearized plasmid was treated with CIP and purified (QiaEx Agarose Gel Extraction). Both, ITR cassette and Rep/Cap sequences were cloned into the multiple cloning site of pTransfer, flanked by *loxP* and FRT sites, in a one-step ligation (1:1:3 DNA ratio, T4 DNA Ligase, overnight at 16 °C) (Fig. 16A). Clones were analyzed by digestions with *Eco* RI and *Sac* I restriction enzymes and sequenced. vi) rHSV/AAV Recombination – HSVQuik System. The HSVQuik system is based on a recombinant HSV-1 BAC cloned genome, the MGH1 (6), a derivative from the $\gamma_1$34.5-deletion mutant R3616 (1). The HSV-1 BAC, fHSVQuik-1, contains: (i) an FRT site upstream and a *loxP* site downstream flanking the BAC backbone, that serve for insertion of the transgene cassette, and later, for removal of the prokaryotic plasmid sequences from the vector

genome; (ii) an EGFP coding sequence in-frame downstream of the truncated ICP6 coding sequence, and a phosphoglycerate kinase (PGK) polyA sequence to facilitate visualization of infected cells; and (iii) an RFP (DsRed1) expression cassette in the middle of the BAC backbone (Fig. 16B) (14). For the construction of the recombinant HSV/AAV hybrid vector the protocol described by Terada et al., 2006 (14) was followed. Briefly, 0.1 µg pTransfer containing the ITR cassette and Rep/Cap sequences together with 0.1 µg pFTP-T helper plasmid (a Flp-expressing plasmid) were electroporated at 25 µF, 1.8 kV, 200Ω into electrocompetent bacteria carrying the fHSVQuik-1 BAC plasmid (14). Cells were recovered in SOC medium containing heat-inactivated chlorotetracycline (20 µg/ml) for 3 h at 30 °C (permissive temperature for the helper pFTP-T, and therefore for the pTransfer shuttle plasmid), with rigorous shaking. Recombinants that had fused at the FRT sites contain the transgene cassette inserted at the UL39 locus of the HSV-1 genome and were selected with 15 µg/ml chloramphenicol and 50 µg/ml ampicilin at 43 °C (restrictive temperature for pFTP-T). After recombination, the prokaryotic plasmid backbones as well as the RFP marker are flanked by the two unidirectional *loxP* sites. Upon cotransfection of the recombinant HSV-1 BAC and a Cre-expressing helper plasmid into Vero cells, the prokaryotic plasmid backbones as well as the RFP marker were excised through Cre-mediated site-specific recombination (Fig. 16C).

Titration of vector stocks

Vero cells were inoculated with recombinant HSV-1. The whole cell and supernatant were harvested 2 to 3 days post-infection, frozen and thawed three times to release intracellular virus, and the lysate was clarified by centrifugation at 3'500 rpm for 10 min at 4 °C. Supernatant was serially diluted 10-fold and added to a monolayer of Vero cells in a 96 well-plate. The cells were inspected daily for cytopathic effects and titers were determined after 5 days (10).

Electronmicroscopy

(i) Negative Staining. Purified virus samples were adsorbed to carbon coated parlodion films mounted on 300 mesh/inch copper grids (EMS, Fort Washington, PA, USA) for 10 min at RT, washed once with destiled water, and stained with 2 % phosphotungstenic acid pH7.0 (Aldrich, Steinheim, Germany) for 1 min. Specimens were analyzed in a transmission electron microscope (CM12, Philips, Eindhoven, The Netherlands) equipped with a CCD camera (Ultrascan 1000, Gatan, Pleasanton, CA, USA) at an acceleration voltage of 100 kV. (ii) Antibody precipitation. Purified virus samples were incubated with an antibody that binds to intact AAV2 particles (Mab, diluted 1:5 in PBS; Research Diagnostics Inc-RDI, Flanders, NJ, clone A20 –RDI-PRO61055) and 0.1 % BSA for 1 h at RT in a

centrifuge adapter. Carbon coated parlodion films mounted on 300 mesh/inch copper grids (EMS, Fort Washington, PA, USA) were placed inside the centrifuge support containing the virus-antibody solution and centrifuged at 5'500 rpm for 30 min at 4 °C (Sorval, rotor SS34). The grids were washed 5 x with PBS + 0.1 % BSA, for 5 min, and incubated with secondary antibody coupled to 12 nm Gold beads (Aurion 1:30 in PBS + 0.1 % BSA). Grids were then washed 5 times with PBS for 5 min and twice with destiled H_2O for 5 min, followed by negative staining.

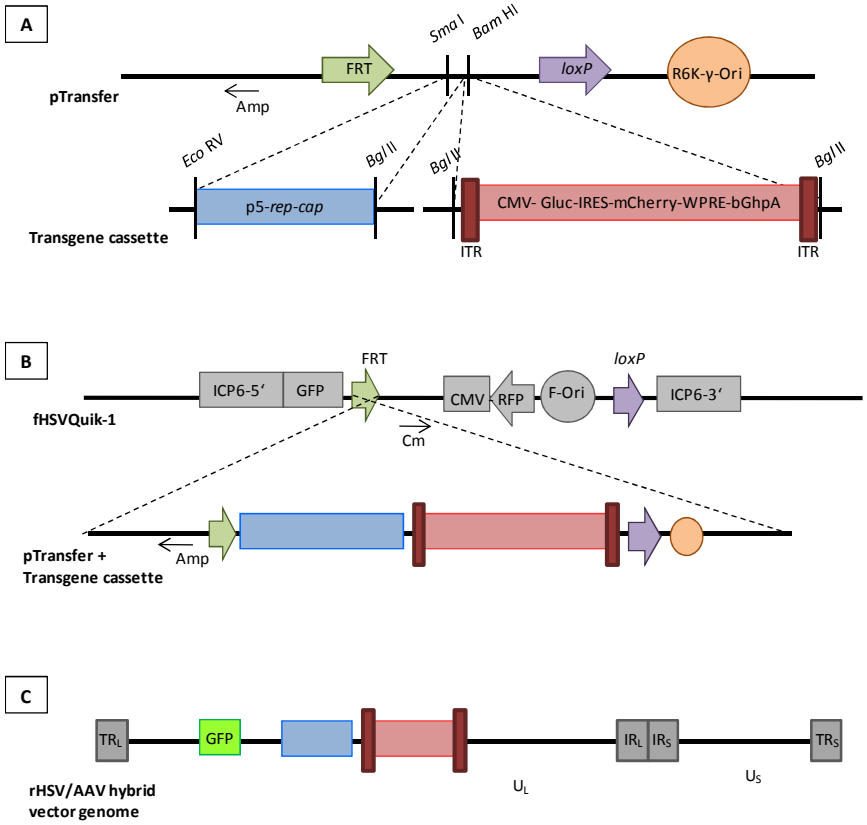

Figure 16. Schematic representation of the rHSV/AAV hybrid vector construction. The construction of a rHSV/AAV hybrid vector that expresses the AAV2 *rep* and *cap* genes, and contains a transgene cassette flanked by the AAV2 ITRs was constructed using the HSVQuik system. First, the transgene cassettes of interest were constructed. Briefly, the ITR cassette, flanked by the AAV2 ITRs, contains the humanized *Gaussia* luciferase (hGluc) reporter gene separated by an internal ribosomal entry site (IRES) from the mCherry sequence both under the control of the CMV promoter, followed by the posttranscriptional regulatory element of Woodchuck Hepatitis virus (WPRE), and a bovine growth hormone polyadenilation signal (bGhpA). The AAV2 *rep* and *cap* genes were PCR amplified. (A) In a one-step ligation both the ITR cassette and the AAV2 *rep-cap* genes were cloned into the multiple cloning site of the pTransfer plasmid flanked by the FRT and *lox*P sites. (B) The insertion of pTransfer into the FRT site of the fHSVQuik-1 BAC plasmid was mediated by flipase (Flp) site-specific recombination in bacteria upon co-electroporation with the helper plasmid pFTP-T. (C) The resulting recombinant HSV/AAV genome encodes a functional GFP marker, and the AAV2 *rep-cap* genes upstream of the AAV2 ITR cassette.

2.3.3.3. Results

Construction of a recombinant HSV/AAV hybrid vector containing an AAV2 ITR-flanked transgene cassette and encoding the AAV2 rep and cap genes

A rHSV/AAV hybrid vector was based on the oncolytic recombinant HSV-1, MGH1, in which both copies of the $\gamma_1$34.5 gene were deleted and an EGFP expression cassette was inserted in-frame downstream of the truncated UL39 (ICP6) gene, therefore restricting the replication in non-dividing cells (e.g. neurons) but maintaining replication on proliferating malignant cells (1, 18).

The rHSV/AAV hybrid vector constructed contained the AAV2 *rep* and *cap* genes, and a transgene cassette flanked by the AAV2 ITRs. The construction of the transgene cassette containing a fluorescent marker flanked by the AAV2 ITRs was described in detail in the Material and Methods. Briefly, the transgene cassette is flanked by the AAV2 ITRs and contains the humanized *Gaussia* luciferase (hGluc) reporter gene under the CMV promoter, separated by an internal ribosomal entry site (IRES) from the red fluorescent protein encoded by the mCherry sequence. In addition, the posttranscriptional regulatory element of Woodchuck Hepatitis virus (WPRE), and a bovine growth hormone polyadenylation sequence (bGhpA) were also inserted into the cassette. For the construction of the cassette encoding the AAV2 Rep and Cap proteins, the AAV2 *rep* and *cap* genes were PCR amplified, and specific restriction enzyme sites were inserted. Both the ITR cassette and the AAV *rep-cap* genes were cloned into the multiple cloning site of pTransfer plasmid in a one-step ligation (Fig. 16A). The shuttle plasmid pTransfer containing the transgenes was inserted into the FRT site of the fHSVQuik-1 BAC plasmid through flipase-mediated site-specific recombination aided by the pFTP-T helper plasmid, as described by Terada et al. (14) (Fig. 16B). The resulting recombinant HSV/AAV genome encodes a functional GFP marker and the AAV2 *rep-cap* genes upstream of the ITR cassette (fHSV/AAV-A1) (Fig. 16C).

In order to verify if the exact recombinations have occurred, the genome of fHSV/AAV-A1 was analyzed. For that, the *Hind* III restriction pattern of BAC plasmid DNA was compared from: (i) the original fHSVQuik-1 clone (fHSVQuik-1), (ii) a recombinant in which only the shuttle plasmid (pTransfer) was inserted (fHSVQuik-pTr), and (iii) the rHSV/AAV BAC clone (fHSV/AAV-A1). The insertion of the pTransfer into the fHSVQuick-1 BAC DNA (fHSVQuik-pTr) divides the 18.5 kb band in three: 12.3, 8.0, and 0.3 kb. The same shift of bands occurred on the fHSV/AAV-A1 construct, but in addition other bands appeared corresponding to (i) the AAV cassette (p5 promoter until the rep68 sequence) (1.7 kb), (ii) the AAV cassette (rep78 to cap sequences), and the ITR cassette (containing the ITR, CMV, and Gluc sequences) (4.0 kb), and (iii) the ITR cassette (containing the mCherry, WPRE, pA) (1.8 Kb). Two small bands of 0.2 kb corresponding to the second ITR and IRES sequences were at

the detection limit. Sequence analysis of the HSV/AAV hybrid BAC DNA construct confirmed the complete insertion of both transgene cassettes into the fHSVQuik vector.

Recombinant HSV/AAV (rHSV/AAV) vectors were rescued by Cre-mediated excision of the prokaryotic plasmid backbone sequences flanked by two unidirectional *loxP* sites. The rescued rHSV/AAV vectors were isolated by serial dilution and plaque purified.

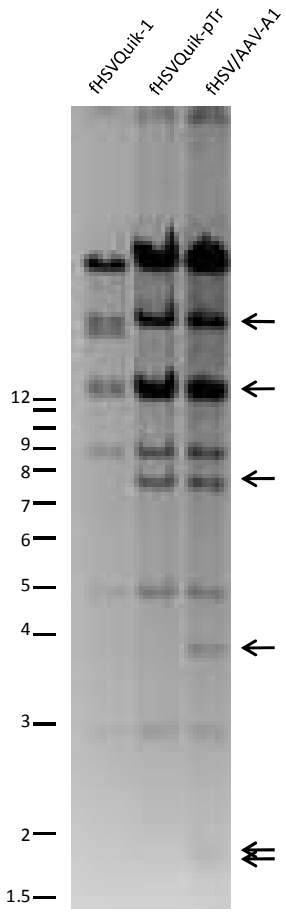

Figure 17. Restriction endonuclease analysis of the rHSV/AAV hybrid BAC DNA construct. The restriction enzyme *Hind* III was used to compare the pattern of the BAC plasmid DNA from the original fHSVQuik-1 clone (fHSVQuik-1), an fHSVQuik in which only the pTransfer plasmid was inserted (fHSVQuik-pTr), and the rHSV/AAV BAC clone containing the AAV2 cassette and the ITR-flanked transgene cassette (fHSV/AAV-A1). Arrows point to the differences between the original and constructed clones.

Partial characterization of the HSV/AAV hybrid vector

The production of rHSV/AAV and rAAV2 particles in rHSV/AAV vector infected cells was evaluated. For this, BHK cells were infected with the rHSV/AAV hybrid vectors and, after 3 days, the infected

culture was harvested and the supernatant clarified and subjected to electron microscopy. The recombinant HSV-1-like particles were observed to contain all structural components: envelope, tegument, and capsid (Fig. 18a, and b). AAV2-like particles were also found in the supernatant of cells infected with the rHSV/AAV hybrid vector (Fig. 18c) and confirmed by immunogold staining with an antibody that specifically binds to intact AAV2 particles (Fig. 18d). Infection of Vero cells with the hybrid vectors resulted in titers of 1.23 x 10^6 PFU/ml.

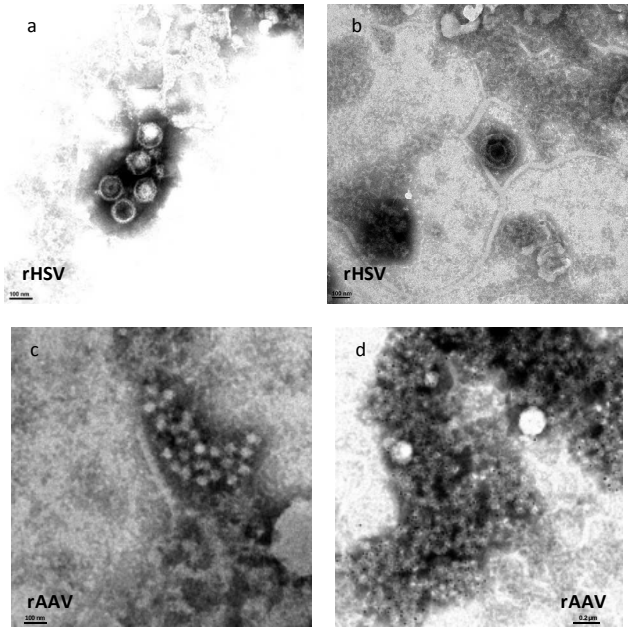

Figure 18. Electron micrographs of rHSV/AAV hybrid vector and rAAV particles. Supernatant of cells infected with rHSV/AAV hybrid vectors was subjected to electron microscopy analysis. (a, b) rHSV-like particles, and (c, d) rAAV-like particles were observed. (d) Immunoprecipitation with an antibody that specifically binds to intact AAV2 particles and immunogold staining confirmed the production of rAAV particles.

2.3.3.4. Conclusions

A rHSV/AAV hybrid vector was constructed based on the oncolytic recombinant HSV-1 vector MGH1 in which the AAV2 elements, and a fluorescent protein marker flanked by the AAV2 ITRs were inserted using the HSVQuik system (14). Upon infection of host cells, rHSV/AAV hybrid vectors were able to generate rAAV2 particles. This suggests that the AAV2 elements encoded by the rHSV/AAV hybrid vector were functional, as rAAV2 particles were shown to assemble. Recombinant HSV-1 particles were also produced, although rHSV/AAV hybrid vectors showed a reduction in 2-logs when compared to titers of wtHSV-1. Further analyses on the inhibitory effects of the AAV2 Rep protein on rHSV-1 replication have to be performed. More detailed characterization of the rHSV/AAV hybrid vector would also include: (i) a replication assay, in order to analyze the amplification/replication of the rHSV vector genomes and of the ITR-flanked transgene cassette; (ii) a site-specific integration assay, to verify the integration of the ITR-flanked transgene cassette into the AAVS1 site of the vector infected cell genome; (iii) evaluation of the infection kinetics of the rHSV/AAV hybrid vectors in gliomas cells and visualization of the extent of rAAV spread *in vitro* and *in vivo*.

Glioblastoma multiforme (GBM) is the most common form of all primary central nervous system neoplasms in adults. The high proliferative and infiltrative nature of these neoplasms results in high recurrence of tumors after the available treatments such as surgery, radiation, and chemotherapy (8, 19). This is demonstrated by the observation that a residual pool of malignant cells surrounding the neurons and blood vessels gives rise to a recurrent tumor that in most of the cases develops adjacent to the resection margin or within several centimeters of the resection cavity. In some cases, satellite lesions may appear due to the migration of tumor cells through the white matter tracts to regions distant from the original tumor mass and even in the contralateral hemisphere (2, 4, 9). As an alternative therapy, the use of oncolytic rHSV-1 for treatment-refractory cancers has been demonstrated with great success in many experimental studies and clinical trials in animals (16). The most used rHSV-1 for clinical efficacy assessment in cancer treatments are the ones lacking both copies of the $\gamma_1$34.5 gene, due to the reported features of replicating exclusively in dividing tumor cells and absence of virulence in experimental animal systems and in humans (1, 12, 18).

In this sense, the rHSV/AAV hybrid vector that launches the production of rAAV particles is a promising candidate as an adjuvant in the treatment of brain tumors. In a model situation, the rHSV/AAV hybrid vector could be used for inoculation directly in the tumor or on the site of tumor resection where it would infect the remaining tumor cells, resulting in the replication of the rHSV/AAV hybrid vector and production of rAAV2 particles. Due to the high efficiency of AAV2 in penetrating solid tumor tissue, such as glioblastomas (15), the rAAV2 particles would be expected to

spread further in the brain tissue reaching distant malignant cells (11, 17). Improvements of this system would include (i) targeting the rAAV particles produced specifically to tumoral cells (3, 5); or (ii) the insertion of a promoter in the transgene cassette carried by the rAAV specific for replication on malignant cells (20).

2.3.3.5. References

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3. Perspectives

Many advances on the knowledge on using viruses as vectors for gene therapy have been occurring, with the development of strategies with promising results. Improvements have been focusing on (i) the combination of advantageous viral features in the construction of hybrid vectors (2–4, 7, 11, 13); (ii) targeting vector particles to specific cell types, via cell-type specific receptors (5, 6) or via engineering of viral capsids (9); (iii) transcriptional targeting using cellular specific promoters (1, 17, 18).

The characteristics of HSV-1, such as a large transgene capacity, neurotropism, and the possibility of constructing oncolytic avirulent mutant viruses confer great potential for the use of this virus as a vector in gene therapy of brain tumors (8, 14–16). AAV due to its lack of human pathogenicity and broad host cell range, is one of the most studied viruses for gene therapy, and currently in use in clinical trials (10, 12). The combination of both HSV-1 and AAV in a hybrid vector would sum up their advantages as an efficient and safe candidate vector when associated with specific-targeting strategies.

The increasing knowledge on virus biology, associated with the development of techniques that allow a better understanding of interactions between the virus and its host will contribute to advances on the design of safer and more efficient viral vectors and vaccines.

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4. Appendix

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Live Visualization of Herpes Simplex Virus Type 1 Compartment Dynamics▽†

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We have constructed a recombinant herpes simplex virus type 1 (HSV-1) that simultaneously encodes selected structural proteins from all three virion compartments—capsid, tegument, and envelope—fused with autofluorescent proteins. This triple-fluorescent recombinant, rHSV-RYC, was replication competent, albeit with delayed kinetics, incorporated the fusion proteins into all three virion compartments, and was comparable to wild-type HSV-1 at the ultrastructural level. The VP26 capsid fusion protein (monomeric red fluorescent protein [mRFP]-VP26) was first observed throughout the nucleus and later accumulated in viral replication compartments. In the course of infection, mRFP-VP26 formed small foci in the periphery of the replication compartments that expanded and coalesced over time into much larger foci. The envelope glycoprotein H (gH) fusion protein (enhanced yellow fluorescent protein [EYFP]-gH) was first observed accumulating in a vesicular pattern in the cytoplasm and was then incorporated primarily into the nuclear membrane. The VP16 tegument fusion protein (VP16-enhanced cyan fluorescent protein [ECFP]) was first observed in a diffuse nuclear pattern and then accumulated in viral replication compartments. In addition, it also formed small foci in the periphery of the replication compartments which, however, did not colocalize with the small mRFP-VP26 foci. Later, VP16-ECFP was redistributed out of the nucleus into the cytoplasm, where it accumulated in vesicular foci and in perinuclear clusters reminiscent of the Golgi apparatus. Late in infection, mRFP-VP26, EYFP-gH, and VP16-ECFP were found colocalizing in dots at the plasma membrane, possibly representing mature progeny virus. In summary, this study provides new insights into the dynamics of compartmentalization and interaction among capsid, tegument, and envelope proteins. Similar strategies can also be applied to assess other dynamic events in the virus life cycle, such as entry and trafficking.

The herpes simplex virus type 1 (HSV-1) virion consists of three different compartments, capsid, tegument, and envelope. The icosahedral capsid has a diameter of 125 nm and contains the virus genome, a double-stranded DNA of 152 kbp. The structural basis of the capsid are the 162 capsomers, which include 150 hexons and 12 pentons (47). The capsomers are connected in groups of three by a complex formed with two copies of VP23 and one copy of VP19c (47, 54, 68). The hexons are composed of six molecules of the major capsid protein VP5. Eleven of the 12 pentons are composed of five molecules of VP5, while 1 of the 12, the so-called portal, is a cylindrical structure of 12 molecules of UL6 (46). Also involved in capsid assembly, but not physical components of the capsids, are the scaffold polypeptides VP22a, VP21, and the serine protease, VP24, which is required for capsid maturation (9, 26, 38, 51). Six copies of VP26, a 12-kDa polypeptide encoded by the UL35 gene, occupy the tips of each hexon and thus decorate the surface of the capsid (42, 69). Although not essential for virus replication in tissue culture, VP26 was demonstrated to be important for infectious virus production in trigeminal ganglia (12). VP26 is a protein expressed later in the virus replication cycle after the onset of DNA replication and has been demonstrated to have multiple phosphorylated forms (43). VP26 has been shown to be recruited in an ATP-dependent manner after pro-capsid formation (8). As it lacks a nuclear localization signal (NLS), it must form complexes with NLS-containing proteins, such as VP5 and VP22a, in order to specifically accumulate in the nucleus (52, 60).

The virus capsid is surrounded by an amorphous layer, the so-called tegument. The tegument contains at least 15 virus-encoded proteins in various copy numbers which play important structural and functional roles during infection (32). One major structural component of the tegument is VP16, a 54-kDa protein encoded by the UL48 gene (63). Although VP16 is not essential for viral DNA replication, its structural role in the tegument is essential. Recombinants of HSV-1 that lack the UL48 gene show impaired replication, a defect in DNA packaging, and the absence of infectious virus progeny (63). VP16 is responsible for transcriptional regulation of immediate-early (IE) genes and is also involved in the modulation of the activities of early and late virus genes (7, 48, 49). VP16 has been shown to coimmunoprecipitate with virion host shutoff protein (55), to cross-link into complexes with gB, gD, and gH (70),

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and to copurify with UL47 (67) and with VP22 (16). Due to its involvement in linking capsid and future envelope-associated tegument proteins during virion formation, VP16 is absolutely required for assembly of infectious virus (63) and plays essential roles in viral maturation and egress (24, 44).

The tegument is surrounded by the viral envelope, which is a lipid membrane of cellular origin that contains at least 11 different viral glycoproteins. The glycoproteins are the major antigenic determinants for the host-specific recognition, and they are involved in cell entry, cell-to-cell spread, and immune evasion. Glycoprotein H, the product of the UL22 gene, is a 110-kDa protein which is essential for infectivity (14) and membrane fusion, but not for receptor binding (22, 25). Glycoprotein H must be coexpressed with gL in order for both proteins to be properly processed, folded, and transported to the virion envelope as well as the infected cell surface (33). The gH/gL complex plays essential roles in viral penetration, cell-to-cell spread, and syncytium formation (53). Both glycoproteins are conserved among the herpesviruses, although some differences regarding assembly, structure, and intracellular transport of the heterocomplex exist between individual herpesviruses (27, 33, 34, 50). Only very recently was it shown that the simultaneous deletion of gH and gB results in a severe deficit in nuclear egress leading to the accumulation of virions in the perinuclear space, whereas the deletion of gH or gB alone did not lead to a significant defect (19). One strategy to investigate mechanisms of viral infection, replication, and assembly is the fusion of viral proteins with autofluorescent proteins (reviewed in reference 5). Many of the HSV-1 proteins have been shown to maintain their functional activity when fused with foreign polypeptides. Among these, a green fluorescent protein (GFP)-VP26 fusion was demonstrated to be incorporated into intranuclear capsids and mature virions, where it was capable of interacting with VP5 while retaining its autofluorescence (13). GFP-VP26 retained its biological activity during the replication cycle, as the recombinant virus replicated at a rate comparable to that of wild-type (wt) virus (13). A recombinant HSV-1 that expressed VP16 fused to GFP (VP16-GFP) also showed normal replication kinetics and incorporation of the fusion protein into the virion (35). Similarly, fusion of enhanced yellow fluorescent protein (EYFP) with gH did not markedly alter gH functions, as the recombinant virus was replication competent and showed stable autofluorescence (39). The EYFP-gH fusion protein formed a stable heterocomplex with gL and was incorporated into the virion envelope as well as cellular membranes (39).

In this study, we have constructed a recombinant HSV-1 that simultaneously encodes the VP26 capsid protein fused with monomeric red fluorescent protein (mRFP), the VP16 tegument protein fused with enhanced cyan fluorescent protein (ECFP), and the gH envelope glycoprotein fused with EYFP. This triple-fluorescent recombinant HSV-1, rHSV-RYC, was replication competent and incorporated the autofluorescent fusion proteins into all three virion compartments. Confocal laser scanning microscopy (CLSM) of living infected cells revealed new insights into the organization and dynamics of HSV-1 infection and into the interactions between HSV-1 virion proteins. To our knowledge this is the first report of the construction and live analysis of a recombinant virus encoding three different autofluorescent fusion proteins.

MATERIALS AND METHODS

Cell culture and virus. BHK, Vero, and Vero 2-2 cells (56) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Penicillin G at 100 units/ml, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B were added to all cell culture media. For culturing Vero 2-2 cells, 500 μg/ml G418 was included in addition.

HSV-1 strain F as well as the recombinant viruses were grown, and titers were determined in Vero cells.

Construction of recombinant viruses. Recombinant HSV-1 encoding one (rHSV-R or rHSV-48Y), two (rHSV-RYor rHSV-RC), or three (rHSV-RYC or rHSV-RYC/2) different virion proteins fused with autofluorescent proteins were generated by homologous recombination in *Escherichia coli* SW102 and *galK* selection/counterselection (62) using a bacterial artificial chromosome (BAC)-cloned HSV-1 strain F genome (pYEbac102; kindly provided by Y. Kawaguchi, Tokyo Medical and Dental University, Japan) (59).

Electroporation and *galK* positive/negative selection. To prepare electrocompetent bacteria, 500 μl of an overnight culture of *E. coli* SW102/YEbac102 was diluted in 25 ml LB medium containing 12.5 μg/ml chloramphenicol in a 50-ml conical flask and grown at 32°C. When the optical density at 600 nm reached 0.6, 10 ml of the culture was transferred to another 50-ml conical flask and incubated at 42°C in a shaking water bath. After exactly 15 min, the culture was briefly cooled on ice, transferred into two 15-ml tubes, and pelleted for 5 min at 5,000 rpm and 0°C. The supernatant was removed, and the pellet was resuspended in 1 ml ice-cold H_2O by gently swirling the tube on ice. Then, 9 ml of ice-cold H_2O was added, and the cells were pelleted again; this step was repeated once more. After the second washing and centrifugation step, the supernatant was removed, and the pellet (approximately 50 μl) was kept on ice until electroporated with PCR product (targeting cassettes). In a first step (*galK* selection), a PCR product (see below) that contained the *E. coli galK* gene flanked by 50 nucleotides of sequence homology to either side of the targeting locus on the virus genome (VP26, gH, and VP16) (Fig. 1) was electroporated into 25 μl of electrocompetent *E. coli* SW102/YEbac102 cells in a 0.1-cm cuvette (Bio-Rad, Hercules, CA) at 25 μF, 1.75 kV, and 200 Ω. After electroporation, the bacteria were grown in 1 ml LB medium for 1 h at 32°C and then washed twice with 1× M9 salts (37 mM Na_2HPO_4, 22 mM KH_2PO_4, 19 mM NaCl) as follows: the culture was pelleted at 13,000 rpm for 15 s, resuspended in 1× M9 salts, and pelleted again. The washing step was repeated once more. After the second wash, the supernatant was removed and the pellet was resuspended in 1× M9 salts before 100-μl aliquots of serial dilutions (1:10, 1:100, and 1:1,000) were plated on galactose minimal medium plates (62) supplemented with 12.5 μg/ml chloramphenicol to select Gal^+ recombinant colonies. After 2 to 3 days of incubation at 32°C, colonies were picked and streaked on MacConkey galactose indicator plates to obtain single bright pink/red Gal^+ colonies. One or two of these colonies were picked to prepare electrocompetent bacteria for the second recombination step, the in-frame introduction of autofluorescent protein-coding sequences into VP26, gH, or VP16 sequences and *galK* counterselection. For this, electrocompetent *E. coli* SW102 cells containing the *galK*-modified YEbac102 were prepared as described above and electroporated with PCR products (targeting cassettes; see below) containing coding sequences of autofluorescent proteins flanked by the same 50 nucleotides present on the *galK* PCR product that target the DNA to homologous sequences in the virus genome (VP26, gH, and VP16). After electroporation, the bacteria were recovered in 10 ml LB medium for 4.5 h at 32°C and washed twice with 1× M9 salts, and serial dilutions were plated on minimal medium plates containing glycerol as carbon source, leucine, biotin, and 2-deoxy-galactose (DOG; Acros Organics, Geel, Belgium) for selection against *galK* (62). After 3 days of incubation at 32°C, colonies were picked, and BAC DNA was isolated and characterized by restriction endonuclease and Southern analysis.

PCR amplification and purification of targeting cassettes. Phusion polymerase (Finnzymes, Espoo, Finland) and the following primers were used to amplify the targeting cassettes: (i) mRFP-VP26 fusion, *galK* selection, ul35-galK-fw, 5′-ACAGCCCTCCCGACCGACACCCCCATATCGCTTCCCGACCTCCGGTCCCG *CCTGTTGACAATTAATCATCGGCA*-3′, and ul35-galK-rev, 5′-CCAAGCGCCCGGACGCTATCGGTGGTAACGGTGCTGGGGCGGTGAAATTG*TCAGCACTGTCCTGCTCCTT*-3′; mRFP-VP26 fusion, *galK* counterselection, ul35-rfp-fw, 5′-ACAGCCCTCCCGACCGACACCCCCATATCGCTTCCCGACCTCCGGTCCCG*ATGGCCTCCTCCGAGGACGTC*-3′, and ul35-rfp-rev, 5′-CCAAGCGCCCGGACGCTATCGGTGGTAACGGTGCTGGGGCGGTGAAATTG*GGCGCCGGTGGAGTGGCGGCC*-3′; (ii) EYFP-gH fusion, *galK* selection, UL22-galK-fw, 5′-GTTATTATTTTGGGCGCTGCGTGGGGTCAGGTCCACGACTGGACTGAGCAG*CCTGTTGACAATTAATCATCGGCA*-3′, and UL22-galK-rev, 5′-CGTGTCGCGCCAGTACATGCGGTCCATGCCCAGGCCATCCAAAAACCATGG*TCAGCACTGTCCTGCTCCTT*-3′; EYFP-gH fusion, *galK* counterselec-

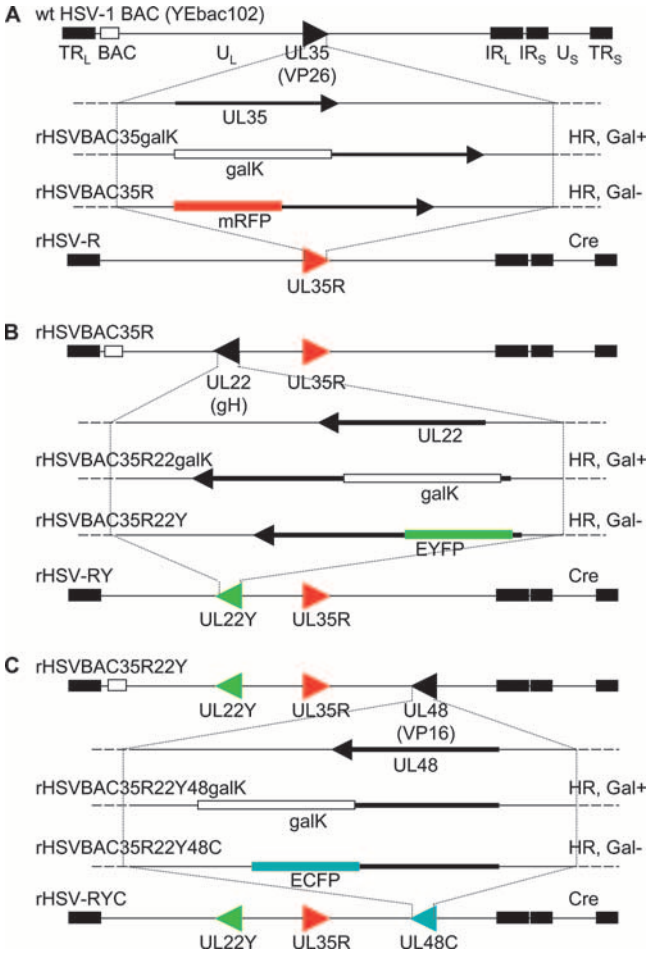

FIG. 1. Construction of rHSV-RYC. (A) Representation of the HSV-1 BAC (YEbac102) genome structure, showing the region containing the U_L35 gene. The *galK* expression cassette was inserted into the U_L35 gene through homologous recombination (HR) and selection for Gal^+ recombinants (rHSVBAC35galK). GalK was then replaced by mRFP coding sequences by HR and counterselected for Gal^- recombinants (rHSVBAC35R). The same procedure was used for the fusion of EYFP with the U_L22 gene on the rHSVBAC35R genome (B) and for the fusion of ECFP with the U_L48 gene on rHSV BAC35R22Y (C). The BAC sequences were removed by the Cre/loxP recombination system (Cre), resulting in the recombinants HSV-1 rHSV-R, rHSV-RY, and rHSV-RYC. TR_L, terminal repeat of the long segment; U_L, unique long segment; IR_L, internal repeat of the long segment; IR_S, internal repeat of the short segment; U_S, unique short segment; TR_S, terminal repeat of the short segment.

tion, UL22-EYFP-fw, 5′-GTTATTATTTTGGGCGCTGCGTGGGGTCAGGTC CACGACTGGACTGAGCAG*GGTGAGCAAGGGCGAGGAGCTGTTC*-3′ and UL22-EYFP-rev, 5′-CGTGTCGCGCCAGTACATGCGGTCCATGCCCAGGC CATCCAAAAACCATGG*CTTGTACAGCTCGTCCATGCCGAG*-3′; (iii) VP16-ECFP fusion, *galK* selection, UL48-galK-fw, 5′-TTCGAGTTTGAGCAGATGTTT ACCGATGCCCTTGGAATTGACGAGTACGGT*CCTGTTGACAATTAATCAT CGGCA*-3′, and UL48-galK-rev, 5′-GGTGACGGGAGGGGAAAACCCAGACGG GGGATGCGGGTCCGGTCGCGCCCC*TCAGCACTGTCCTGCTCCTT*-3′; VP16-ECFP/EYFP fusion, *galK* counterselection, UL48-ECFP-fw, 5′-TTCGAGTTTGAG CAGATGTTTACCGATGCCCTTGGAATTGACGAGTACGGT*GTGAGCA AGGGCGAGGAGCTGTTC*-3′, and UL48-ECFP-rev, 5′-GGTGACGGGAGG GGAAAACCCAGACGGGGGATGCGGGTCCGGTCGCGCCCC*TTACTTG TACAGCTCGTCCATGCC*-3′. Sequence portions shown in italics are homologous to the *galK* gene. The following plasmids (10 ng) were used as templates: pgalK for all galK selection/targeting cassettes (obtained from S. Warming, National Cancer Institute, Frederick, MD) (62), pcDNA-mRFP-N1 (obtained from U. F. Greber, University of Zurich, Zurich, Switzerland) for the VP26-mRFP targeting cassette, and pECFP-N1 and pEYFP-N1 for amplification of VP16-ECFP/EYFP and gH-EYFP targeting cassettes, respectively. The PCR conditions were as follows: 94°C for 15 s, 60°C for 30 s, and 72°C for 1 min, for 30 cycles. After completion of the reaction, DpnI (10 U; New England Biolabs, Allschwil, Switzerland) was added for digestion of the template for 2 h. The DpnI-digested reaction mix was run on a 1% agarose gel, and the PCR product was purified using a QIAQuick PCR purification kit (Qiagen, Hombrechtikon, Switzerland) followed by ethanol precipitation. The DNA was resuspended in 40 μl H_2O, and an aliquot of 2 to 5 μl (10 to 30 ng) was used for electroporation.

Excision of BAC sequences and isolation of recombinant HSV-1. To excise the BAC DNA backbone and isolate recombinant viruses, 1.2×10^6 Vero 2-2 cells (56) per 6-cm tissue culture plate were cotransfected with 0.2 μg of plasmid p116, which expresses Cre recombinase with an NLS (kindly provided by K. Tobler, University of Zurich, Zurich, Switzerland), and 2 μg of CsCl gradient-purified recombinant HSV-1 BAC DNA using Lipofectamine (Invitrogen). After 2 to 3 days of incubation at 37°C, the supernatant was harvested and plaque purified twice, and the excision of the BAC sequences was verified by PCR.

Virus replication assays. For the determination of growth kinetics, Vero cells were inoculated at a multiplicity of infection (MOI) of 0.1 or 5 PFU per cell. After 2 h of incubation at 37°C, 5% CO_2, the cultures were washed three times with PBS and then incubated with DMEM containing 2% FBS. Samples (cell culture medium and cells) were removed after 0, 12, 24, 36, and 48 h. The cell culture medium was removed from the cells and serially diluted to determine the titers on Vero cells. The titers of cell-associated virus were determined on Vero cells inoculated with serial dilutions of supernatants of cells prepared by three cycles of freezing and thawing, followed by centrifugation at $1,900 \times g$.

Determination of particle/PFU ratios. To determine the particle counts, virus stocks with known titers (PFU/ml) were mixed with 204-nm-diameter latex beads (Agar Scientific, Essex, United Kingdom) of known concentration, adsorbed onto 300-mesh parlodion- and carbon-coated copper electron microscope grids for 5 min at room temperature (RT), briefly washed with H_2O, and negatively stained with 2% sodium phosphotungstate, pH 7.4, for 1 min at RT. Samples were examined in a transmission electron microscope (CM12; Philips, Eindhoven, The Netherlands), and the relative numbers of virus particles and latex beads were determined, which allowed us to calculate the absolute numbers of virus particles in the virus stocks.

Purification of virions. Virions were purified from BHK or Vero cells infected at an MOI of 0.1 PFU with either wt HSV-1, rHSV-RYC, or rHSV-RYC/2. When the cytopathic effect (CPE) was complete, the cultures were frozen and thawed three times, and cell debris was removed by centrifugation for 10 min at $2,600 \times g$ and 4°C. Virions were purified through 60%, 30%, and 10% sucrose (in PBS) gradients in Beckman Ultra-Clear 25- by 89-mm centrifuge tubes, which were centrifuged for 2 h at 28,000 rpm and 4°C using a Beckman SW28 rotor. The interface between the 30% and 60% sucrose layers was collected, diluted in PBS, and ultracentrifuged for 1 h at 25,000 rpm and 4°C. Following resuspension of the pellet in Hanks' buffered saline solution, the virion stocks were frozen in a dry ice-ethanol bath and stored at −80°C.

Immunoprecipitation, SDS-PAGE, Western analysis, and silver staining. Vero cells (4×10^5 cells per well in a 12-well plate) were mock infected or infected with either wt HSV-1, rHSV-RY, rHSV-RYC, or rHSV-RYC/2 at an MOI of 1 PFU. When CPE was almost complete (between 24 and 48 h postinfection [p.i.]), the cells were washed with cold PBS and prepared for immunoprecipitation or directly lysed with sodium dodecyl sulfate (SDS) loading buffer, boiled for 5 min, and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using the antibodies listed below. For immunoprecipitation, the cells were lysed with 200 μl of EBC170 lysis buffer (50 mM Tris pH 8.0, 170 mM NaCl, 0.5% NP-40) supplemented with one tablet of protease inhibitor cocktail (Complete, mini, EDTA-free; Roche Diagnostics, Rotkreuz, Switzerland) per 10 ml. The cell extract was collected and immunoprecipitated with the gH-specific monoclonal antibody (MAb) LP11 (kindly provided by A. Minson and H. Browne, University of Cambridge, United Kingdom) (6) diluted 1:30 in EBC170. After 1 h at 4°C, complexes were allowed to attach to protein A-Sepharose beads. After washing the beads four times with EBC170 buffer, the LP11 MAb complexes were eluted from the beads by adding SDS loading buffer. The samples were boiled for 5 min, and proteins were separated by SDS-PAGE and transferred onto nylon membranes (Protran; Whatman, Bottmingen, Switzerland). Nonspecific reactions were blocked by incubating the membranes for 1 h with PBS containing 5% skimmed milk and 0.3% Tween 20. Membranes were then incubated for 1 h with antibodies against VP26 (rabbit polyclonal antibody [PAb] diluted 1:1,000 in PBS, 0.3% Tween 20 [PBS-T]; kindly provided by A. Helenius, ETH Zurich, Switzerland), VP16 (MAb LP1, diluted 1:500 in PBS-T;

kindly provided by A. Minson and H. Browne, University of Cambridge, United Kingdom) (41), GFP (MAb JL-8BD, diluted 1:8,000 in PBS-T; Clontech, Saint-Germain-en-Laye, France), ICP4 (MAb, diluted 1:10,000 in PBS-T; Advanced Biotechnologies, Columbia, MD), ICP8 (MAb clone 10A3, diluted 1:10,000 in PBS-T; Abcam, Cambridge, United Kingdom), and VP22 (rabbit PAb AGV031, diluted 1:10,000 in PBS-T; kindly provided by G. Elliott, Marie Curie Research Institute, Oxted, United Kingdom). After washing, the membranes were incubated with rabbit anti-mouse immunoglobulin G (IgG)–horseradish peroxidase (HRP) (1:10,000 in PBS-T; Sigma-Aldrich, Buchs, Switzerland) or goat anti-rabbit IgG–HRP (1:10,000 in PBS-T; Sigma-Aldrich, Buchs, Switzerland). Target proteins were visualized by enhanced chemiluminescence (ECL Western blotting analysis system; GE Healthcare, Zurich, Switzerland) and autoradiography (Lumi-film chemiluminescent detection film; Roche Diagnostics, Rotkreuz, Switzerland). A molecular weight standard (BenchMark prestained ladder; GIBCO, Invitrogen, Basel, Switzerland) was used to determine the sizes of the protein bands. Silver staining of gels was performed using the Bio-Rad silver stain kit (Bio-Rad, Hercules, CA) according to the manufacturer's manual.

CLSM. CLSM was performed on a Leica TCS SP2 AOBS confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) equipped with an incubation chamber (THE BOX; Live Imaging Services, Reinach, Switzerland), a temperature control device (THE CUBE; Live Imaging Services, Reinach, Switzerland), and a gas mixer (THE BRICK; Live Imaging Services, Reinach, Switzerland). The settings for the individual fluorophores were as follows: 4′,6-diamidine-2′-phenylindole (DAPI), excitation at 405 nm and recording at 415 to 480 nm; ECFP, excitation at 458 nm and recording at 468 to 510 nm; fluorescein isothiocyanate (FITC), excitation at 488 nm and recording at 498 to 570 nm; EYFP, excitation at 514 nm and recording at 520 to 550 nm; mRFP/Atto590/Alexa Fluor 594 (AF594), excitation at 594 nm and recording at 604 to 700 nm. In order to avoid channel overlap, blue and red channels were recorded simultaneously while the yellow/green channels were recorded separately. The images shown in Fig. 7 and 8, below, were deconvolved with a blind deconvolution algorithm using the Huygens Essential 2.6.0p1 software (Scientific Volume Imaging, Hilversum, The Netherlands). Image processing was done with Imaris 5.0.1 (Bitplane AG, Zurich, Switzerland) and Adobe Photoshop CS 8.0.1 software. The detailed procedures were as follows.

(i) High-resolution CLSM of live or fixed, infected cells. Vero cells were seeded on Lab-Tek four-well chambered coverglasses (Nalge Nunc International, Rochester, NY) at 10^5 cells/well. On the following day, the cells were washed once with cold (4°C) DMEM and incubated for 15 min at 4°C, and the DMEM was replaced with cold (4°C) virus inoculum at the MOIs described in the figure legends. The viruses were then allowed to adsorb to the cells for 1 h at 4°C with gentle shaking. Subsequently, the virus inoculum was replaced with warm (37°C) Iscove's modified Dulbecco's medium (GIBCO, Invitrogen, Basel, Switzerland) supplemented with 25 mM HEPES and 2% FBS, and the cells were incubated at 37°C, 5% CO_2. At the indicated times after infection (temperature shift), live cells were observed by CLSM in a humidified atmosphere at 37°C, 5%CO_2. Where mentioned in the text below, the cells were fixed with 3.7% formaldehyde in PBS for 15 min at RT prior to microscopy.

(ii) Time-lapse CLSM of live, infected cells. Vero cells were seeded into 35-mm glass-bottom dishes (MatTek, Ashland, MA) at 5×10^5 cells/dish. On the following day, the cells were infected with rHSV-RY or rHSV-RYC diluted in DMEM at the MOIs described in the text below. The viruses were allowed to adsorb for 2 h at 37°C, 5% CO_2. The cells were then washed with PBS, overlaid with Iscove's modified Dulbecco's medium supplemented with 25 mM HEPES and 2% FBS, and incubated at 37°C, 5% CO_2. At the indicated time, live cells were observed by CLSM in a humid atmosphere at 37°C, 5% CO_2. Images were recorded at intervals of 15 to 25 min.

Immunofluorescence. Vero cells were seeded on round 12-mm coverglasses in 24-well plates at 10^5 cells/well. On the following day, the cells were washed with PBS and infected with wt HSV-1 or rHSV-RY diluted in DMEM at the MOIs described in the figure legends. The viruses were allowed to adsorb for 1 to 2 h at 37°C, 5% CO_2. The cells were then washed with PBS, overlaid with DMEM supplemented with 2% FBS, and incubated at 37°C, 5% CO_2. At the indicated time points, the cells were washed once with cold PBS and fixed with 3.7% formaldehyde in PBS for 15 min at RT, and the fixation was stopped with 0.1 M glycine in PBS for 5 min at RT. Immunofluorescence staining, DAPI staining, and embedding of cells were performed as described previously (29) except that the cells were permeabilized with 0.2% Triton X-100 in PBS for 15 min at RT and that 0.2 mg/ml human IgG (Sigma-Aldrich, Buchs, Switzerland) was included in the blocking solution when cells were stained with antibodies of rabbit origin. Primary antibodies were used at the following dilutions: anti-HSV-1 ICP8 MAb 7381 (kindly provided by R. D. Everett, MRC Virology Unit, Glasgow, United Kingdom), 1:500 (see Fig. 6C, below) or 1:1,000 (see Fig. 6D, below); anti-HSV-1 gH MAb LP11, 1:3; anti-HSV-1 VP16 MAb LP1, 1:3 (see Fig. 8A, below) or 1:200 (see Fig. 8D, below); rabbit anti-HSV-1 VP26 PAb VP26/C (kindly provided by P. Desai, Johns Hopkins University, Baltimore, MD) (12), 1:400. Secondary antibodies were used as follows: $F(ab')_2$ fragment of goat anti-rabbit IgG(H+L)–AF594 (Molecular Probes, Invitrogen, Basel, Switzerland), 1:200; goat anti-rabbit IgG(H+L)–FITC (Southern Biotechnology, Birmingham, AL), 1:100; goat anti-mouse IgG(H+L)–AF594 (Molecular Probes, Invitrogen, Basel, Switzerland), 1:200; Fab fragment of goat anti-mouse IgG(H+L)–FITC (Jackson ImmunoResearch, West Grove, PA), 1:100.

FISH. Infection with wt HSV-1 and immunofluorescence staining for VP26 were performed as described above. Fluorescent in situ hybridization (FISH) was performed as described previously (18) with the following modifications: (i) cells were fixed with precooled methanol for 10 min at −20°C; (ii) immunofluorescence staining was performed before the hybridization step; (iii) fluorescent labeling of BAC-cloned HSV-1 DNA (YEbac102) was performed with an Atto590 nick translation labeling kit according to the manufacturer's manual (Jena Bioscience, Jena, Germany).

Electron microscopy. Vero cells were grown on sapphire disks for 2 days prior to infection with rHSV-RYC. After 24 h, cells were fixed with 0.25% glutaraldehyde for 30 min and then frozen in a high-pressure freezer (HPM010; BAL-TEC Inc., Balzers, Liechtenstein) as previously described (37). The sapphire disks carrying the frozen cells were transferred into a freeze-substitution unit (FS 7500; Boeckeler Instruments, Tucson, AZ) precooled to −88°C for substitution with acetone and subsequent fixation with 0.25% glutaraldehyde and 0.5% osmium tetroxide at temperatures between −30°C and +2°C as previously described in detail (64) and embedded in Epon at 4°C. Sections of 50 to 60 nm were analyzed in a transmission electron microscope (CM12; Philips, Eindhoven, The Netherlands) equipped with a slow-scan charge-coupled-device camera (Gatan, Pleasanton, CA) at an acceleration voltage of 100 kV.

RESULTS

Construction of a recombinant HSV-1 encoding mRFP-VP26, VP16-ECFP, and EYFP-gH fusion proteins. The goal of this study was to construct and characterize a recombinant HSV-1 that simultaneously encodes selected structural proteins from all three virion compartments fused with autofluorescent proteins, in particular, capsid protein VP26 fused with mRFP, tegument protein VP16 fused with ECFP, and envelope glycoprotein H fused with EYFP. This triple-fluorescent recombinant HSV-1 was constructed via homologous recombination in *E. coli* using a BAC-cloned HSV-1 strain F genome (pYEbac102) (59) and the *galK* positive/negative selection method (62) as described in Materials and Methods. Briefly, in a first step, a DNA fragment containing the *galK* expression cassette flanked by homology arms that target the cassette between codons 1 and 5 of the VP26 coding sequences was electroporated into *E. coli* SW102 cells that contained the pYEbac102 HSV-1 BAC DNA. Gal-positive recombinant bacteria were selected on galactose minimal medium plates. BAC DNA prepared from these clones was characterized by restriction endonuclease analysis (not shown), and one clone (rHSVBAC35galK) was selected for the second recombination step. For the second step, the *galK* expression cassette was replaced by electroporating a DNA fragment containing the mRFP expression cassette, again flanked by the homology arms that facilitate the in-frame insertion of mRFP coding sequences between codons 1 and 5 of the VP26 coding sequences, into *E. coli* SW102/rHSVBAC35galK. After *galK* counterselection with DOG, Gal-negative colonies were picked, BAC DNA was prepared and characterized by restriction endonuclease analysis (not shown), and one clone (rHSV BAC35R; Fig. 1A) was selected for further manipulation. The subsequent fusion of gH with EYFP (rHSVBAC35R22Y; Fig. 1B) and VP16 with ECFP (rHSVBAC35R22Y48C; Fig. 1C)

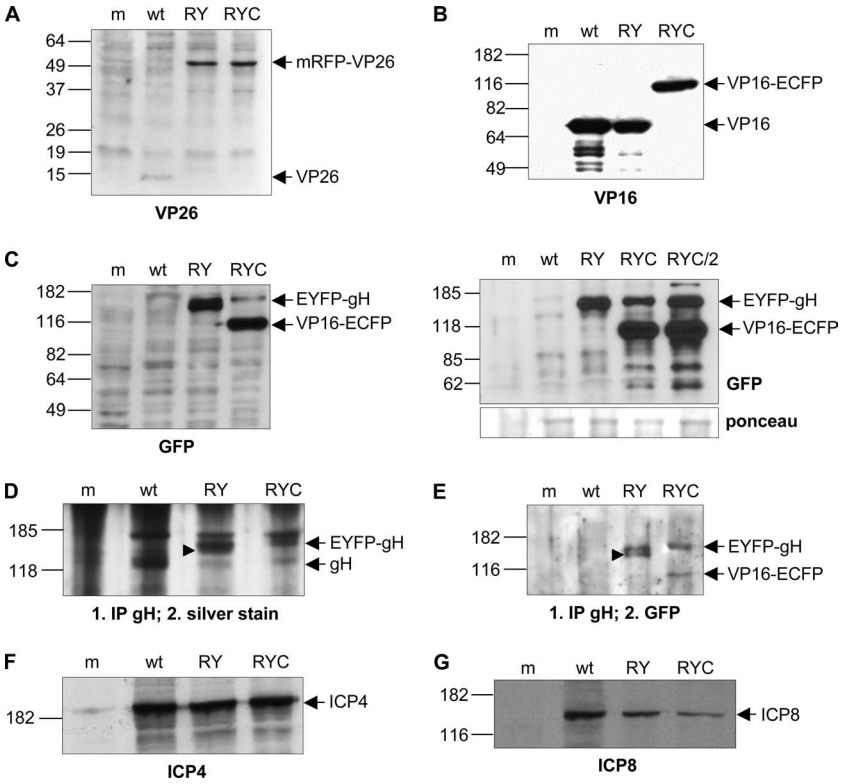

FIG. 2. Expression of fluorescent fusion proteins in infected cells. Vero cells were mock infected (m) or infected with either wt HSV-1 (wt), rHSV-RY (RY), rHSV-RYC (RYC), or rHSV-RYC/2 (RYC/2) at an MOI of 1 PFU and harvested when the CPE was approximately 90%. Cell lysates were analyzed by SDS-PAGE followed by Western blotting with antibodies against VP26 (A), VP16 (B), GFP (C), ICP4 (F), and ICP8 (G). For detection of gH and EYFP-gH, cell lysates were immunoprecipitated with a gH-specific antibody followed by SDS-PAGE and silver staining (D) or Western blotting with a GFP-specific antibody (E). Arrows indicate the mRFP-VP26, VP16-ECFP, and EYFP-gH fusion proteins, as well as the wt VP26, VP16, gH, ICP4, and ICP8 proteins. The arrowheads in panels D and E point to a band that likely represents the EYFP-gH precursor. Sizes of molecular weight standards are indicated.

was performed using the same *galK* positive/negative selection procedure described above and in Materials and Methods. The final recombinant, rHSVBAC35R22Y48C, contained codons 1 to 225 of mRFP fused to codons 5 to 108 of VP26, codons 2 to 239 of EYFP inserted between codons 6 and 9 of gH, and codons 1 to 489 of VP16 fused to codons 2 to 239 of ECFP. Two additional recombinant HSV-1 BACs were constructed that contained either codons 1 to 489 of VP16 fused to codons 2 to 239 of EYFP (rHSVBAC48Y) or codons 1 to 225 of mRFP fused to codons 5 to 108 of VP26 and codons 1 to 489 of VP16 fused to codons 2 to 239 of ECFP (rHSVBAC35R 48C). A second triple-fluorescent recombinant HSV BAC, rHSVBAC35R22Y48C/2, was created by introducing codons 2 to 239 of EYFP between codons 6 and 9 of gH in rHSVBAC 35R48C. The two triple-fluorescent HSV BACs, rHSVBAC 35R22Y48C and rHSVBAC35R22Y48C/2, encode the same fusion proteins, but these were inserted in different orders. To confirm that the recombinations had occurred as expected, BAC DNA was analyzed by restriction endonuclease digestion and Southern blot analysis (not shown). Finally, to rescue recombinant viruses and release the virus genome from the BAC backbone, recombinant HSV-1 BAC DNA was cotransfected with a Cre recombinase-expressing plasmid, p116, into mammalian cells (Fig. 1). Progeny virus was harvested after 2 to 3 days and plaque purified twice, and the excision of the BAC sequences was confirmed by PCR analysis (not shown). The recombinant viruses constructed and used in this study were designated as follows: rHSV-R (mRFP-VP26), rHSV-48Y (VP16-EYFP), rHSV-RY (mRFP-VP26 and EYFP-gH), rHSV-RC (mRFP-VP26 and VP16-ECFP), rHSV-RYC (mRFP-VP26, EYFP-gH, and VP16-ECFP), and rHSV-RYC/2 (mRFP-VP26, EYFP-gH, and VP16-ECFP). The two triple-fluorescent recombinants, rHSV-RYC and rHSV-RYC/2, produced comparable titers and showed identical patterns of fluorescence in infected cells (not shown).

Synthesis of autofluorescent fusion proteins in infected cells. To verify that the recombinant viruses expressed the fluorescent fusion proteins, Vero cells were mock infected or infected with either wt HSV-1, rHSV-RY, or rHSV-RYC at an MOI of 1 PFU per cell. When the CPE was approximately 90%, cells were harvested and analyzed. The 90% CPE was reached approximately 24 h p.i. for the wt virus and 48 h p.i. for the recombinant virus, consistent with delayed replication kinetics of the recombinant viruses (see Fig. 3, below). Western blotting with a rabbit anti-VP26 PAb (kindly provided by A. Helenius, ETH, Zurich, Switzerland) detected the 12-kDa VP26 protein from wt HSV-1-infected cell lysates. In the lysates of rHSV-RY- or rHSV-RYC-infected cells, the 12-kDa VP26 was not detected but was replaced with a band of approximately 50 kDa, which corresponds to the size expected for the mRFP-VP26 fusion protein (Fig. 2A).

The band intensity of the wt 12-kDa VP26 protein was consistently weaker than that of the 50-kDa mRFP-VP26 fusion protein. The reason for this might be that the relatively small VP26 protein adsorbed less well to the nitrocellulose membrane than the larger fusion protein.

Western blotting with the anti-VP16 MAb LP1 (41) detected the VP16 protein in lysates of cells infected with either wt HSV-1 or rHSV-RY. In rHSV-RYC-infected cell lysates, however, the native VP16 was replaced by a band of approximately 100 kDa, which was also detected by the GFP-specific MAb JL-8BD (Fig. 2B and C). As the GFP-specific antibody reacts not only with GFP and ECFP but also with EYFP, a band of approximately 140 kDa was visible in both rHSV-RY- and rHSV-RYC-infected cell lysates which represented the EYFP-gH fusion protein (Fig. 2C, left panel). Of note is that the levels of the EYFP-gH fusion protein appeared to be higher in rHSV-RY- than in rHSV-RYC-infected cells. We therefore compared the EYFP-gH expression level of rHSV-RYC to that of the independently constructed triple-labeled virus, rHSV-RYC/2, as well as to rHSV-RY. As shown in Fig. 2C (right panel), the ratios between EYFP-gH and VP16-ECFP were comparable for both triple-labeled viruses. In addition, in this experiment, the total amounts of EYFP-gH appeared similar for the double- and triple-labeled viruses. The reason for the fairly low levels of EYFP-gH detected in some experiments therefore appears to be due to variations in the efficiency of infection between different experiments, rather than due to an inherent inability of the triple-colored viruses to express normal amounts of EYFP-gH.

To compare the expression levels of gH from wt HSV-1 and those of EYFP-gH from the recombinant viruses, cell lysates were immunoprecipitated with the gH-specific MAb LP11 (6), which binds to a conformational epitope of gH, followed by SDS-PAGE and silver staining of the gel (Fig. 2D). A band corresponding to the molecular mass of gH (110 kDa) was visible for wt HSV-1 but was replaced with bands corresponding to the molecular mass of EYFP-gH (140 kDa) in the samples of the recombinant viruses. The levels of gH appeared to be higher for wt HSV-1 and for rHSV-RY than for rHSV-RYC. To confirm expression of the EYFP-gH fusion protein, lysates were first immunoprecipitated with the gH-specific MAb LP11 and then analyzed by SDS-PAGE and Western blotting. The GFP-specific antibody detected the approximately 140-kDa EYFP-gH fusion protein in both rHSV-RY- and rHSV-RYC-infected cell lysates (Fig. 2E). Of note is that the levels of EYFP-gH were similar in this setting. Interestingly, in rHSV-RY-infected cell lysates but not in rHSV-RYC-infected cell lysates, a slightly smaller band was also visible, which likely represents the EYFP-gH precursor (30) (Fig. 2E). The accumulation of the EYFP-gH precursor might be a consequence of the higher total levels of EYFP-gH of rHSV-RY resulting in incomplete processing of the precursor, while the lower EYFP-gH levels expressed by rHSV-RYC may be completely processed into the mature form. The GFP-specific antibody also detected the VP16-ECFP fusion protein in rHSV-RYC-infected and LP11-immunoprecipitated cell lysates (Fig. 2E), which is consistent with an earlier finding that gH can coimmunoprecipitate with VP16 (31). An alternative explanation, however, could be that VP16-ECFP and EYFP-gH coimmunoprecipitate because GFP-derived proteins can undergo weak dimerization (57, 65, 66).

We also compared the accumulation of the HSV-1 IE protein ICP4 and the early protein ICP8 among wt HSV-1 and the recombinants. While ICP4 (Fig. 2F) accumulated to comparable levels in cells infected with either wt HSV-1, rHSV-RY, or rHSV-RYC, the levels of ICP8 (Fig. 2G) were reduced in cells infected with the recombinant viruses. In conclusion, these results demonstrated that the fusion proteins mRFP-VP26, VP16-ECFP, and EYFP-gH were expressed in infected cells with ongoing compromised levels of gene expression.

Growth kinetics of autofluorescent recombinant HSV-1. We assessed the growth properties of recombinants that expressed the mRFP-VP26 fusion alone or coexpressed two or three of the fusion proteins. In the first set of experiments, Vero cells were infected at a low MOI (0.1 PFU) with either wt HSV-1, rHSV-R, rHSV-48Y, rHSV-RY, rHSV-RC, or rHSV-RYC, and cultures were harvested at 12, 24, 36, and 48 h p.i. as described in Materials and Methods. Virus yields in both cell culture supernatant (Fig. 3A) and cells (Fig. 3B) were titrated separately (PFU/ml). The growth properties of the recombinant viruses compared to wt HSV-1 can be summarized as follows: (i) the kinetics of virus release was delayed for recombinants rHSV-RY and rHSV-RYC, while all the other recombinants showed kinetics similar to that of wt HSV-1 (Fig. 3A); (ii) in the supernatant, the final titers of the recombinant viruses were reduced 2.6-fold (rHSV48Y) to 40-fold (rHSV-RYC) at 48 h p.i; (iii) in the cell pellet, the final titers of the recombinant viruses were reduced 7.7-fold (rHSV-RY) to 62-fold (rHSV-RC) at 48 h p.i. While it appeared that the fusion of EYFP to gH contributed to the delayed kinetics of virion release of rHSV-RY and rHSV-RYC (Fig. 3A), the contribution of the individual fusions to the reduced final titers was less obvious; specifically, the fusion of EYFP to VP16 alone (rHSV-48Y) had the weakest effect on the final titers, and all other recombinants, whether single, double, or triple labeled, showed similar final titers in the supernatants (Fig. 3A).

In a second set of experiments, we compared the growth properties of rHSV-RYC and wt HSV-1 at a high MOI (5 PFU) (Fig. 3C and D). Under these conditions, the growth deficit of the triple-labeled recombinant was less pronounced. Although the delayed kinetics of virus production was still observed, the final titers at 48 h p.i. differed only by a factor of 5.8 in the supernatant and a factor of 5.6 in the pellet. These findings suggest that the growth deficit of rHSV-RYC can be partially overcome by infection at high MOI, which obviates the need for efficient cell-to-cell spread.

To further characterize the growth properties of the recombinant viruses, we determined the particle/PFU ratios of two recombinants, rHSV-RY and rHSV-RYC, as well as wt HSV-1. We observed particle/PFU ratios of 68 ± 49 (mean $\pm$ standard deviation) for rHSV-RY, 26 ± 19 for rHSV-RYC, and 1.3 ± 0.9 for wt HSV-1, indicating that the recombinant viruses were on average 20- to 50-fold less infectious than the wt virus, a finding which at least partially explains the reduced titers in the recombinant virus stocks.

In summary, the recombinant viruses showed reduced titers both in the supernatant as well as in the pellet, with more pronounced reductions at low than at high MOI. While the fusion of EYFP to gH seemed to contribute to a delay in the

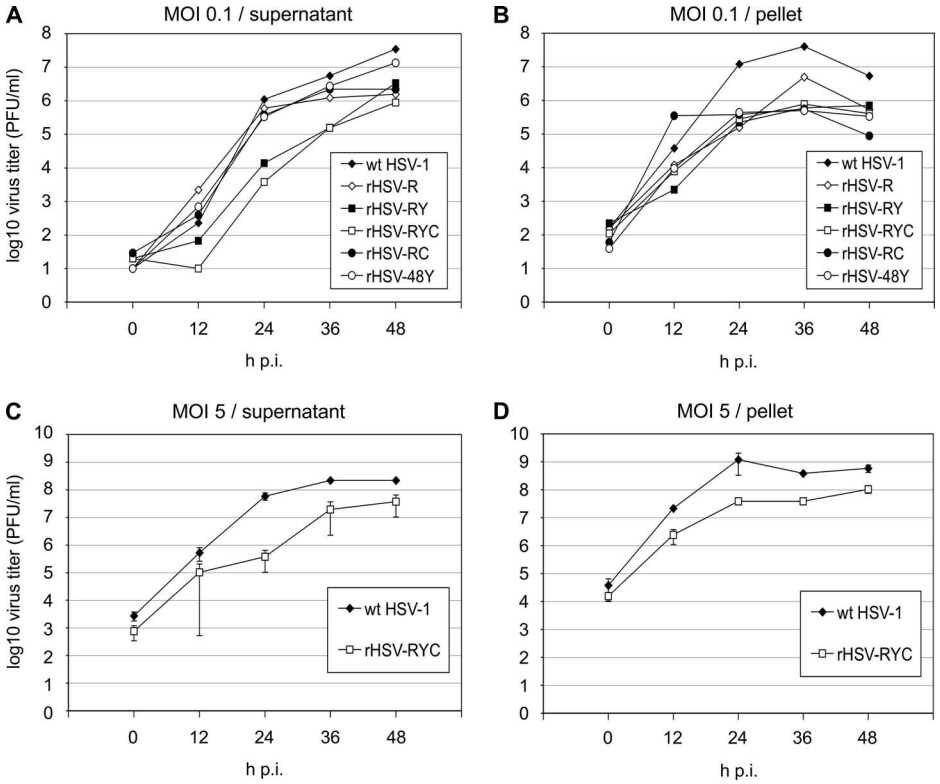

FIG. 3. Growth kinetics of recombinant HSV-1 and wt HSV-1. Vero cells were infected with MOIs of 0.1 (A and B) or 5 (C and D) PFU of either a recombinant HSV-1 (rHSV-R, rHSV-RY rHSV-RYC, rHSV-RC, or rHSV-48Y) or wt HSV-1, and progeny virus was harvested from the cell culture medium (A and C) or from the cells (B and D) at 0, 12, 24, 36, and 48 h p.i. Titers are expressed as PFU per ml. The titers represent means from three experiments. Error bars represent standard deviations.

kinetics of virion release, there was no clear correlation between the number of tagged virion proteins and the reduction in titer. Finally, the recombinant viruses did not seem to have a specific deficit at the level of virion egress, since the differences between the wt virus and the recombinants were comparable in the supernatant and the pellet. The data rather suggest that the kinetics of recombinant virus replication was delayed.

Incorporation of autofluorescent fusion proteins into the virion. To confirm that all three fusion proteins were incorporated into the virion, wt HSV-1 virions and rHSV-RYC virions were prepared by sucrose gradient centrifugation and analyzed for the presence of the recombinant proteins. Western blotting with the VP26-specific PAb revealed the 12-kDa VP26 in wt HSV-1 virions and the 50-kDa mRFP-VP26 fusion protein in rHSV-RYC virons (Fig. 4A). Similar to the observations made in Fig. 2A, the band intensity of the wt VP26 protein was weaker than that of the mRFP-VP26 fusion protein. As previously mentioned, this difference might be due to different efficiencies of adsorption to the nitrocellulose membrane, since it is unlikely that the fusion protein is incorporated more efficiently into the virions than the wt protein. Similar observations were also made in a previous report (45). In order to obtain a visible band for wt VP26, a doubled amount of wt virions was loaded for the blot shown in Fig. 4A. Western blotting with the VP16-specific MAb revealed the presence of VP16 in the wt virions and VP16-ECFP in the rHSV-RYC virions, the amounts of which appeared comparable (Fig. 4B). The VP16-ECFP fusion protein, but not wt VP16, could also be detected with the GFP-specific MAb (Fig. 4C). As expected, the anti-GFP MAb also detected the EYFP-gH fusion protein (Fig. 4C). As noted in Fig. 2, the ratio of EYFP-gH to VP16-ECFP was quite variable between individual experiments (Fig. 2C, compare left and right panels). To detect gH from wt HSV-1 and EYFP-gH from rHSV-RYC, virion proteins were immunoprecipitated with the gH-specific MAb LP11 followed by SDS-PAGE and silver staining. The results shown in Fig. 4D demonstrate the presence of gH in wt virions and EYFP-gH in rHSV-RYC virions. Again, it appeared that the level of gH was higher in the wt virus than in with the recombinant. As expected, immunoprecipitation of virion lysates with LP11 followed by Western blotting with the anti-GFP MAb detected the EYFP-gH fusion protein but not the wt gH (Fig. 4E). As previously noted (Fig. 2E), VP16-ECFP coimmunoprecipitated with EYFP-gH (Fig. 4E). Staining for the VP22 tegument protein confirmed that comparable amounts of virions were loaded on the gels (Fig. 4F). Taken together, these results demonstrate that all three fusion proteins, mRFP-VP26, VP16-ECFP, and EYFP-gH, are indeed incorporated into the virus particle, although the amounts of incorporated EYFP-gH were lower than those of wt gH.

Dynamics of compartmentalization of mRFP-VP26, VP16-ECFP, and EYFP-gH in infected cells. Next, we monitored the localization of mRFP-VP26, VP16-ECFP, and EYFP-gH in

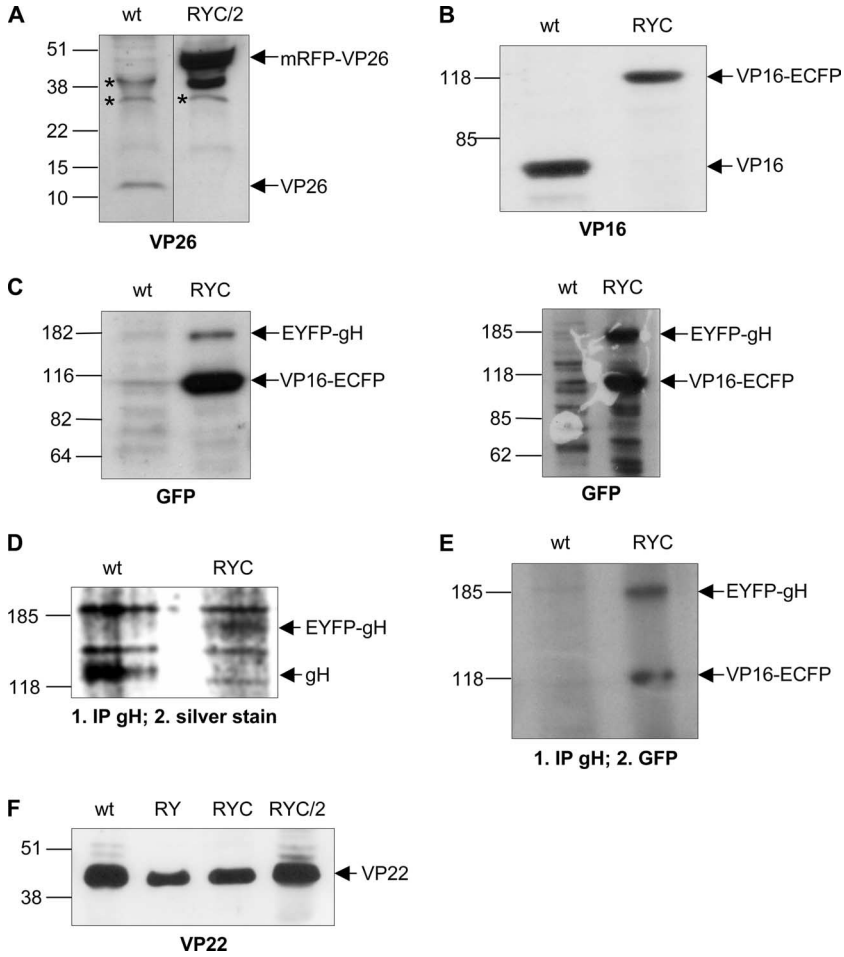

FIG. 4. Incorporation of fluorescent proteins into virions. Purified wt HSV-1 (wt), rHSV-RY (RY), rHSV-RYC (RYC), and rHSV-RYC/2 (RYC/2) were analyzed by SDS-PAGE followed by Western blot analysis with antibodies specific for VP26 (A), VP16 (B), GFP (C), and VP22 (F). For detection of gH and EYFP-gH, virion proteins were immunoprecipitated with a gH-specific antibody and analyzed by SDS-PAGE followed by silver staining (D) or Western blotting with a GFP-specific antibody (E). The corresponding fusion proteins mRFP-VP26, VP16-ECFP, and EYFP-gH, as well as the wt VP26, VP16, gH, and VP22 proteins are indicated. Unspecific bands are marked by asterisks. Sizes of molecular weight markers are shown.

infected cells. For this, Vero cells were plated onto chambered coverglasses and infected with rHSV-RYC, and live cells were examined by CLSM at various times after infection. In the first hours postinfection, small fluorescent foci were detected on the surface of the cells and within the cytoplasm (Fig. 5a to d). Some foci show colocalizations of ECFP, EYFP, and mRFP signals, suggesting that they represent intact virions from the virus inoculum (Fig. 5a to d, insets). De novo protein synthesis was first observed at 6 h p.i. for VP16-ECFP, which was found diffusely in the nucleus with accumulation in very early viral replication compartments (RCs) (Fig. 5e and h). At 8 h p.i., VP16-ECFP was present in mature nuclear RCs as well as in the cytoplasm, where it was found in a diffuse pattern with accumulation in some small perinuclear foci (Fig. 5i and l). At this time, expression of EYFP-gH and mRFP-VP26 also became apparent. In particular, EYFP-gH was found diffusely in the cytoplasm with some accumulation in a vesicular pattern around the nucleus (Fig. 5j and l), while mRFP-VP26 was found in small microfoci which were distributed throughout the nucleus with accumulation in viral RCs (Fig. 5k and l). These small microfoci displayed a high mobility within the nucleus (not shown), reflecting active movement of nuclear capsids (21). The localization of the VP26 and VP16 fusion proteins to RCs was confirmed by staining rHSV-R- and rHSV-48Y-infected cells for ICP8, a marker for RCs (Fig. 6C and D). Between 10 and 16 h p.i., VP16-ECFP was predominantly found in the viral RCs as well as in small foci in their periphery. In addition, the cytoplasmic VP16-ECFP accumulation became more intense, with a pronounced recruitment of VP16-ECFP to the plasma membrane, where it was found in a dot-like pattern (Fig. 5m and p). At this stage, EYFP-gH was still prominent in the cytoplasm but now strongly accumulated in the nuclear membrane (Fig. 5n and p). Microfoci of mRFP-VP26 were still observed in the nucleus, but in addition, mRFP-VP26 was also observed in much larger foci which preferentially formed in the periphery of the RCs, but it did not colocalize with the small VP16-ECFP foci (Fig. 5o and p). These large foci have previously been suggested to correspond to sites of capsid assembly, so-called assemblons (13). Between 18 and 24 h p.i., two additional patterns became apparent.

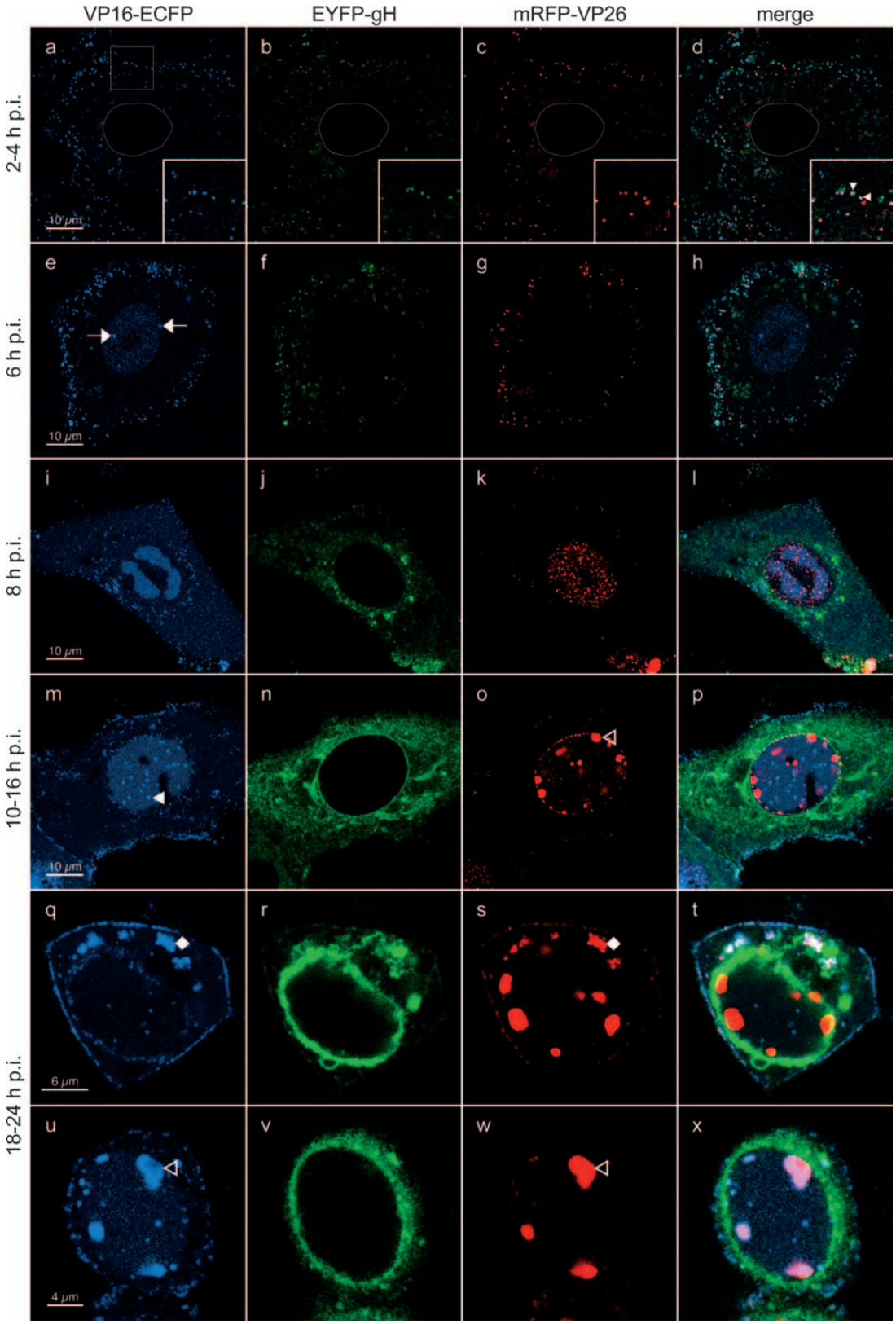

FIG. 5. High-resolution CLSM of living rHSV-RYC-infected cells. Vero cells were infected with an MOI of 18 PFU, and live cells were observed by CLSM with settings specific for ECFP (VP16-ECFP fusion protein), EYFP (EYFP-gH fusion protein), and mRFP (mRFP-VP26 fusion protein). The thin gray lines in panels a to d mark the contours of the nucleus. The insets in panels a to d show a magnification of the sector denoted by the white square. The filled arrowheads within the insets point to colocalizations of ECFP, EYFP, and mRFP signals. Arrows, early RCs; filled triangle, VP16-ECFP foci in periphery of RCs; open triangles, large mRFP-VP26 and VP16-ECFP foci in periphery of nuclei; filled diamonds, asymmetric, perinuclear accumulation of VP16-ECFP and mRFP-VP26. Images represent single *z* stacks of the cells.

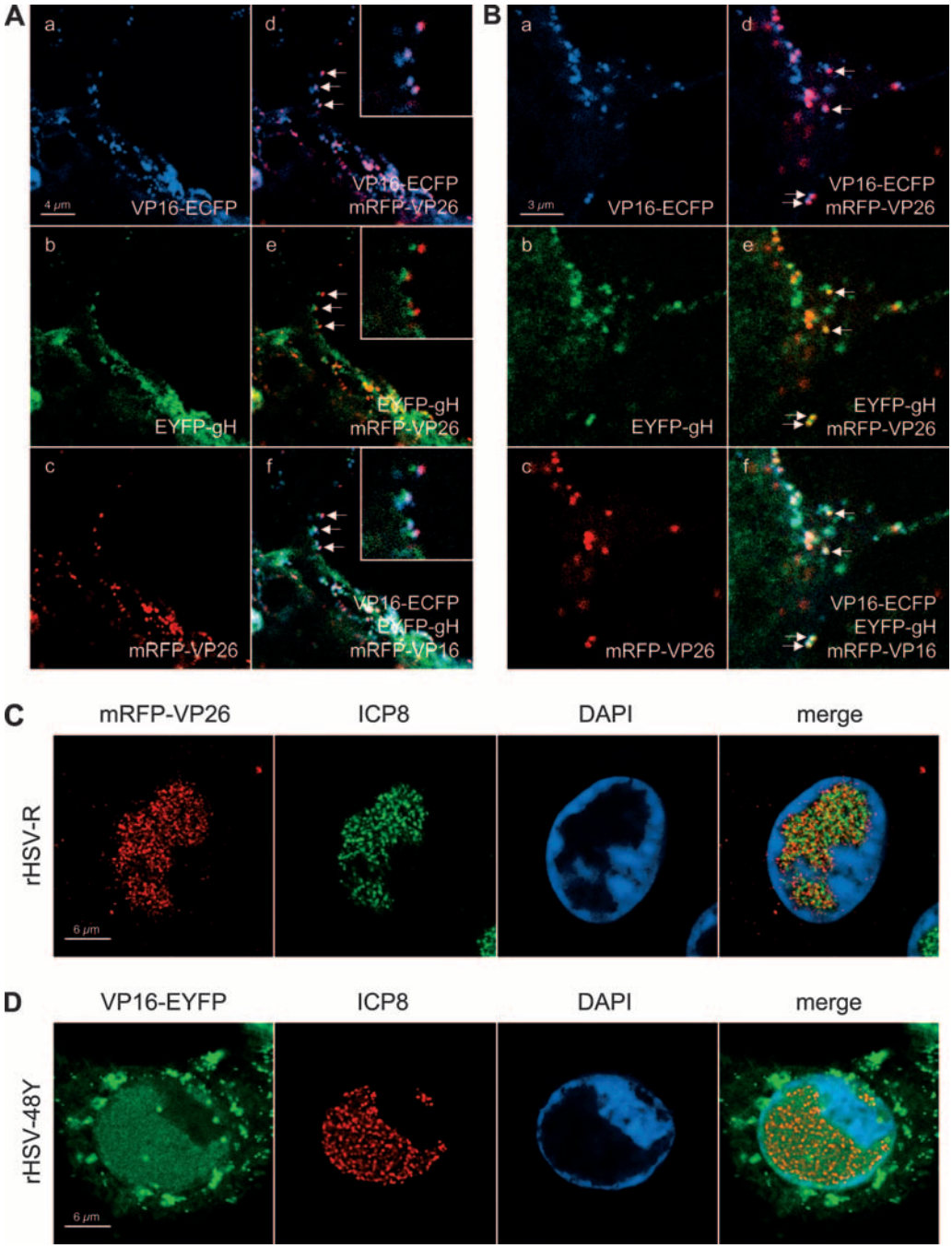

FIG. 6. High-resolution CLSM of rHSV-RYC-infected cells and rHSV-R- and rHSV-48Y-infected cells stained for ICP8. (A) Vero cells were infected with rHSV-RYC, and living cells were observed by CLSM as described for Fig. 5. The images show a high magnification of a protrusion of the plasma membrane (top of picture). The arrows point to foci in which all three fusion proteins colocalize. The insets show magnifications of these foci. (B) Vero cells were infected with rHSV-RYC, and fixed cells were observed by CLSM as described for panel A. (C) Vero cells were infected with rHSV-R at an MOI of 10 PFU, fixed at 12 h p.i., and stained with the anti-ICP8 MAb 7381 and a FITC-conjugated secondary antibody, as well as DAPI. The cells were observed by CLSM with settings specific for DAPI, FITC (ICP8), and mRFP (mRFP-VP26 fusion protein). (D) Vero cells were infected with rHSV-48Y at an MOI of 10 PFU and stained as described for panel C, except that an AF594-conjugated secondary antibody was used. The cells were observed by CLSM with settings specific for DAPI, EYFP (VP16-EYFP fusion protein), and AF594 (ICP8). Images in panels A to D represent single *z* stacks of the cells.

First, the majority of the VP16-ECFP was consistently recruited out of the nucleus into the cytoplasm. Specifically, it was found accumulating in an asymmetric, perinuclear pattern reminiscent of the Golgi complex, where it often colocalized with mRFP-VP26 and associated with EYFP-gH (Fig. 5q to t). Second, in a subset of cells, VP16-ECFP was recruited into the large mRFP-VP26 foci in the periphery of the nucleus, while EYFP-gH remained in a pronounced perinuclear pattern (Fig.

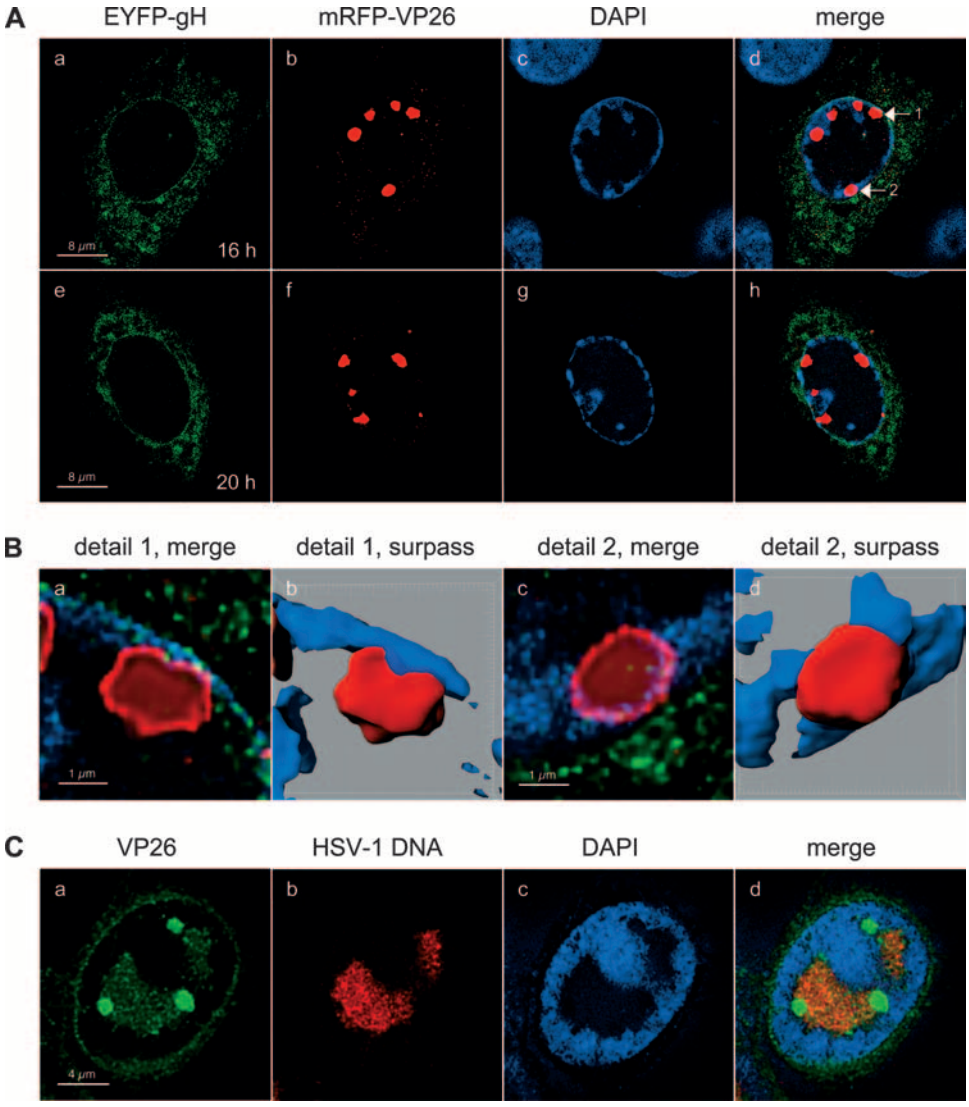

FIG. 7. High-resolution CLSM of large VP26 foci. (A) Vero cells were infected with rHSV-RY at an MOI of 0.3 PFU, fixed at the indicated times postinfection, stained with DAPI, and observed by CLSM with settings specific for DAPI, EYFP (EYFP-gH fusion protein), and mRFP (mRFP-VP26 fusion protein). Images represent single *z* stacks of the cells. (B) Panels a and c show magnifications of the mRFP-VP26 foci marked with the numbered arrows shown in panel A, while panels b and d show surpass views of three-dimensional reconstructions of the same foci. (C) Vero cells were infected with wt HSV-1 at an MOI of 10 PFU, fixed at 12 h p.i., and stained with the rabbit anti-VP26 PAb VP26/C and a FITC-conjugated secondary antibody. HSV-1 DNA was detected by FISH using an Atto590-labeled probe. Cells were observed by CLSM with settings specific for DAPI, FITC (VP26), and Atto590 (HSV-1 DNA). Images represent single *z* stacks of the cells.

5u to x). Finally, at these late time points, the dot-like accumulations of VP16-ECFP at the plasma membrane consistently colocalized with EYFP-gH and mRFP-VP26 (Fig. 5q to t), suggesting that they reflect the accumulation of progeny virus at the plasma membrane. This possibility was further investigated by high-magnification CLSM of the plasma membrane, which, as previously described, often formed pronounced plasma membrane protrusions (35) (Fig. 6A and B). On such plasma membrane protrusions, we observed a very strong association of VP16-ECFP and mRFP-VP26 (Fig. 6A, panel d) as well as a somewhat looser association of EYFP-gH with mRFP-VP26 and VP16-ECFP (Fig. 6A, panels e and f). The looser association of EYFP-gH with mRFP-VP26 and VP16-ECFP is probably due to the fact that we observed living cells and that while the ECFP and the mRFP channels were recorded simultaneously, the EYFP channel was recorded separately, thus explaining the observed differences in the degree of colocalization. Indeed, the association between the three signals was more pronounced in fixed cells (Fig. 6B). These observations, together with the fact that the number of dots increased in the course of infection, support the hypothesis that the dot-like accumulation of virion proteins of three different compartments at the plasma membrane might correspond to accumulating virion progeny. Nevertheless, it cannot be excluded that at least a subset of these foci also represent virions from the inoculum which did not successfully enter the cell and thus remained bound to the plasma membrane.

In order to obtain a more detailed picture of the subnuclear

distribution of the large mRFP-VP26 foci, we infected cells with rHSV-RY, fixed them at 16 or 20 h p.i., and stained the nuclei with DAPI. CLSM analysis revealed that, at such late time points, the large mRFP-VP26 foci were often located at the very periphery of the nuclei (Fig. 7A) and that the density of mRFP-VP26 within these large foci was not homogenous (Fig. 7B). Rather, mRFP-VP26 accumulated strongly at the rim and displayed only a weak accumulation in the interior, suggesting a hollow structure of these foci (Fig. 7B). In addition, we observed that while some of the VP26 structures were still separated from the nuclear membrane by a thin layer of condensed chromatin (Fig. 7B, panels a and b), others displaced the chromatin to such an extent that they virtually gained access to the nuclear membrane, which was visible by virtue of EYFP-gH fluorescence (Fig. 7B, panels c and d). In order to test if these large VP26 foci corresponded to an accumulation of DNA-filled capsids, we codetected VP26 and HSV-1 DNA in wt HSV-1-infected cells (Fig. 7C). While VP26 and HSV-1 DNA colocalized within the RCs, the large VP26 foci did not contain HSV-1 DNA. Assuming that DNA within virions is accessible for FISH, these data suggest that the large VP26 foci do not correspond to accumulations of DNA-filled capsids.

To ascertain that the distribution of the fluorescent virion proteins corresponded to that of the respective wt proteins, we performed infections with wt HSV-1 and detected VP16, gH, and VP26 by immunofluorescence. CLSM analysis of stained cells revealed that the patterns for all three wt virion proteins were very similar to those observed with the recombinant viruses (Fig. 8A to C). However, it has to be noted that the accumulation of VP16 in large foci at the nuclear periphery and their colocalization with VP26 was not observed in wt HSV-1-infected cells stained with VP26- and VP16-specific antibodies (Fig. 8D). This analysis also demonstrated that the structure of the large VP26 foci was identical to that observed with the mRFP-VP26 fusion protein (Fig. 8C, panel h). Interestingly, the proportion of cells displaying such large VP26 foci appeared to be somewhat smaller in wt HSV-1-infected cells than in cells infected with the recombinant viruses.

Time-lapse CLSM of cells infected with either rHSV-RYC or rHSV-RY allowed us to obtain a dynamic view of the interaction between VP16, gH, and VP26 in the infected cells (Fig. 9; see also movies S1 to S3 in the supplemental material). Specifically, the image series demonstrated a very dynamic compartmentalization of VP16-ECFP in the course of infection. While VP16-ECFP initially steadily accumulated in the nuclear RCs and later in the small foci in the periphery of the RCs, there was a very rapid and pronounced recruitment of VP16-ECFP out of the nucleus into the cytoplasm and, in some cells, into the large mRFP-VP26 foci at the periphery of the nucleus in a late stage of infection. This redistribution was fairly rapid and generally occurred within 1 to 2 h (Fig. 9A). In addition, the time-lapse series revealed the progressive accumulation of EYFP-gH at the nuclear membrane and in the perinuclear cytoplasmic pattern (Fig. 9A and B). Finally, the analysis demonstrated that the initially small foci of mRFP-VP26 expanded and coalesced to form much larger foci, which were pushed toward the periphery of the nucleus as infection progressed (Fig. 9A and B).

Ultrastructural analysis of rHSV-RYC-infected cells. Electron microscopy revealed clear indications for normal maturation of the triple-fluorescent recombinant HSV-1, rHSV-RYC, with identical phenotypes to wt HSV-1 (Fig. 10). These phenotypes include formation of capsids with occasional crystalline-like accumulation within the nucleus (Fig. 10C), budding of capsids at the inner nuclear membrane (Fig. 10B), budding of capsids at Golgi membranes (Fig. 10D), virions within vacuoles (Fig. 10E), and virions in the extracellular space (Fig. 10F).

DISCUSSION

Autofluorescent proteins have been used extensively to study many different biological processes, including the replication cycle of viruses (reviewed in reference 5). Recombinant herpesviruses encoding individual virus proteins fused with autofluorescent proteins have previously been constructed to study several different aspects of the herpesvirus life cycle, including trafficking, assembly, and maturation (11, 13, 15, 17, 20, 35, 39). Here, we report for the first time the construction and time-lapse analysis of a recombinant HSV-1 that simultaneously encodes virus proteins from three different virion compartments, capsid (VP26), tegument (VP16), and envelope (gH), fused with different autofluorescent proteins (mRFP, ECFP, and EYFP, respectively).

Crucial to the construction of the triple-fluorescent recombinant HSV-1 was the availability of a BAC-cloned HSV-1 genome (59), which facilitated the easy manipulation of the virus genome in bacteria. The combination of the HSV-1 BAC with the possibility for both positive and negative selection in bacteria provided by the *galK* system (62) allowed the serial introduction of the three fusion genes into the virus genome. The reconstitution of recombinant virus progeny and the elimination of the BAC backbone were accomplished following cotransfection of the recombinant HSV-1 BAC DNA with a Cre recombinase-expressing plasmid in mammalian cells (Fig. 1).

Western analysis of infected cells demonstrated that all three fusion proteins were synthesized (Fig. 2). Interestingly, the triple-fluorescent recombinant HSV-1, rHSV-RYC, was replication competent, although with delayed kinetics, and incorporated all three fusion proteins into the different virion compartments. It appeared that the fusion of EYFP to gH was responsible for a delay in the kinetics of virus release (Fig. 3). This is consistent with a previous report describing the fusion of EYFP to the N terminus of gH (39). Although the authors of that study contended that the recombinant virus replicated to titers comparable to those of wt HSV-1, it has to be noted that the wt virus used in their study (strain 17 syn^+) reached only a titer of 5.25×10^6 PFU/ml, which is rather low for a wt virus. The fusion of autofluorescent proteins to VP16 and VP26 also seemed to affect virus titers, although there were no indications that fusion to the essential VP16 would have a greater impact on viral replication fitness than fusion to the nonessential VP26. In addition, the analysis did not support the notion that the effects of the individual fusions were additive. Rather it appeared that the addition of at least one autofluorescent fusion protein to a virus protein resulted in a somewhat delayed kinetics of virus production (gH) or reduced

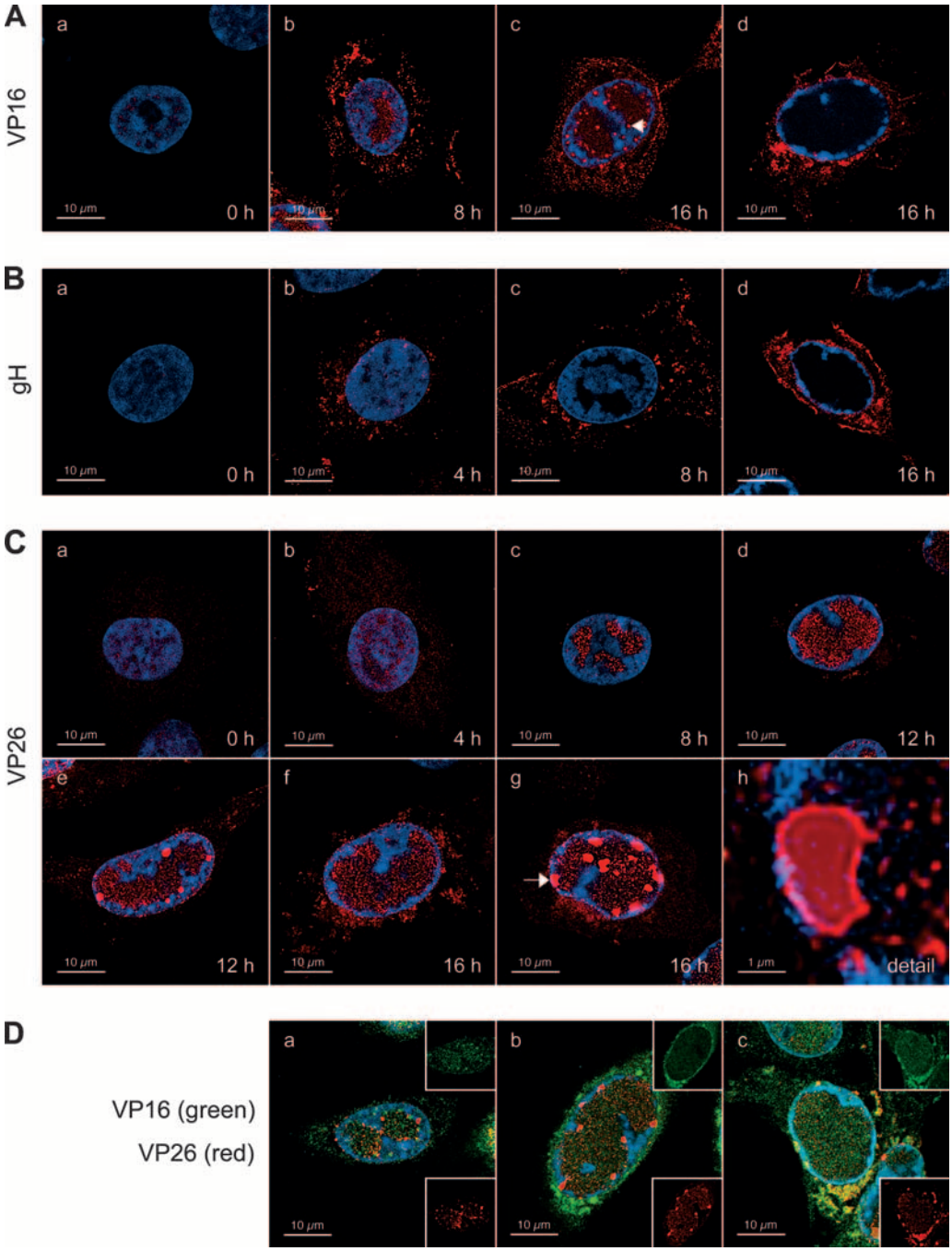

FIG. 8. Immunofluorescence staining for VP16, gH, and VP26 in wt HSV-1-infected cells. (A) Immunofluorescence staining for VP16. Vero cells were infected with wt HSV-1 at an MOI of 10 PFU. At the indicated times postinfection, the cells were fixed and stained with the anti-VP16 MAb LP1 and an AF594-conjugated secondary antibody, as well as DAPI. The cells were then observed by CLSM with settings specific for DAPI and AF594. Filled arrowhead, VP16 foci in the periphery of RCs. (B) Immunofluorescence staining for gH. Vero cells were infected and stained as described for panel A, except that the anti-gH MAb LP11 was used. (C) Immunofluorescence staining for VP26. Vero cells were infected and stained as described for panel A, except that the rabbit anti-VP26 PAb VP26/C was used. Panel h shows a magnification of the VP26 focus marked with an arrow. (D) Vero cells were infected as described for panel A, fixed at 16 h p.i., and stained with DAPI, the anti-VP16 MAb LP1 (detected with an AF594-conjugated secondary antibody), and the rabbit anti-VP26 PAb VP26/C (detected with a FITC-conjugated secondary antibody). The cells were then observed by CLSM with settings specific for DAPI, AF594 (VP16, shown in green), and FITC (VP26, shown in red). Images in panels A to D represent single *z* stacks of the cells.

final titers (VP16 or VP26). Electron microscopy analysis revealed that the triple-fluorescent recombinant HSV-1 was comparable to wt HSV-1 also at the ultrastructural level (Fig. 10). Moreover, CLSM of infected cells demonstrated that the distribution and compartmentalization of the three autofluorescent fusion proteins encoded by rHSV-RYC were comparable to those observed with either wt HSV-1 or recombinants of HSV-1 that encode autofluorescent proteins fused with

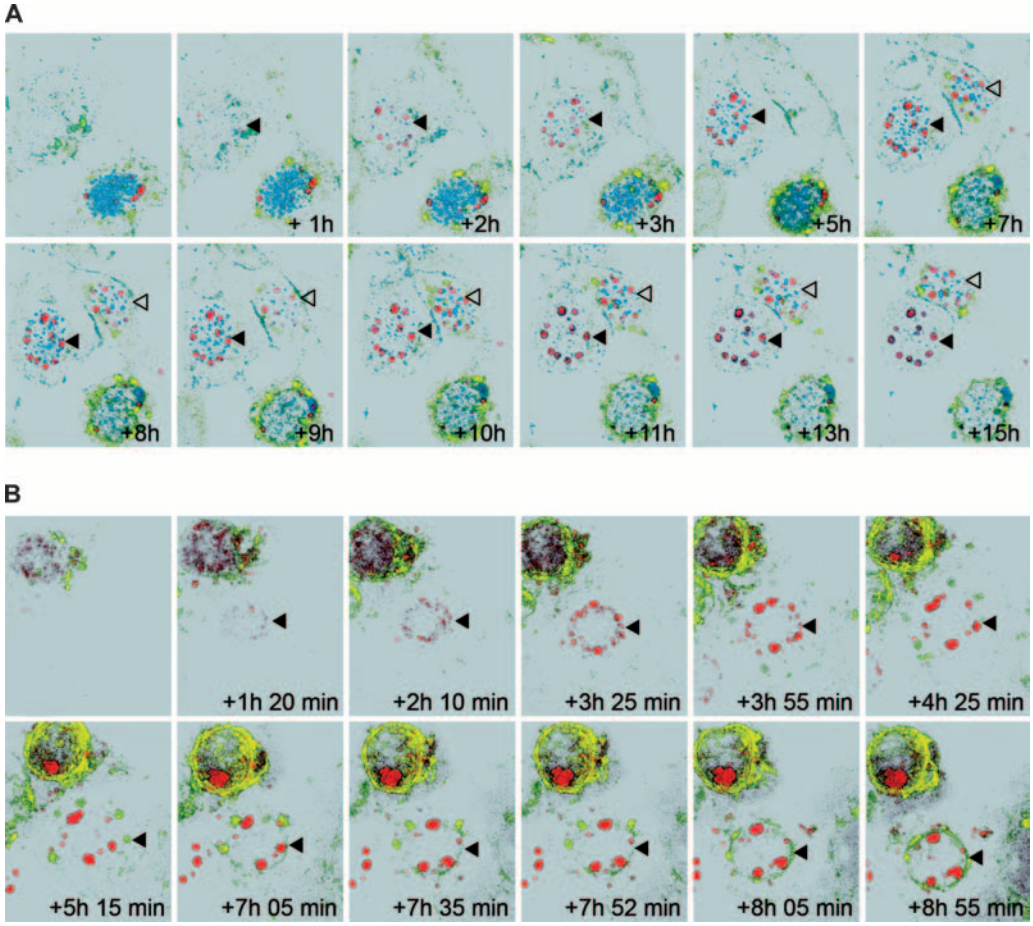

FIG. 9. Time-lapse CLSM of rHSV-RYC- and rHSV-RY-infected cells. Vero cells were infected with rHSV-RYC (A) or rHSV-RY (B) at MOIs of 2 and 0.3, respectively. Cells that just started to accumulate fluorescent proteins were monitored by CLSM with settings specific for ECFP (VP16-ECFP), EYFP (EYFP-gH), and mRFP (mRFP-VP26). Selected frames at the indicated intervals are shown. Images were processed with Imaris software in the surpass view mode. Arrowheads denote the cells described in the text.

VP26, gH, or VP16 individually (Fig. 5 to 8) (13, 35, 39). Finally, the strong dot-like association of autofluorescent fusion proteins of three different virion compartments, VP16-ECFP, mRFP-VP26, and EYFP-gH, at plasma membrane protrusions (Fig. 6A and B) supports the hypothesis that these dots indeed are mature virions and that progeny virions incorporate sufficient amounts of the fluorescent fusion proteins to allow their visualization, although this has to be confirmed on the ultrastructural level. Nevertheless, it would be less convincing to draw such a conclusion with a recombinant HSV-1 that encodes only one or two autofluorescent virion proteins.

The major advantage of our approach is the possibility for simultaneously visualizing the distributions and interactions between different virus proteins and virion compartments on the single cell level. Moreover, our strategy allows study of the dynamics of these events in live cells. For example, mRFP-VP26 and VP16-ECFP colocalized within RCs in the nucleus but, in addition, they both also formed foci in the periphery of the RCs that did not colocalize (Fig. 5). In a previous report it was hypothesized that the VP16 foci in the periphery of the RCs may correspond to sites where capsids acquire VP16 (35). Our experiments, however, suggest that this hypothesis is unlikely. In the course of the infection, the mRFP-VP26 foci expanded and coalesced to form larger foci that relocated to the periphery of the nucleus. In the majority of the cells, VP16-ECFP was redistributed from the nucleus to the cytoplasm late in infection, in a process that occurred very rapidly (generally within 1 to 2 h). Of note is that in a subset of cells, VP16-ECFP was, in addition, recruited into the large mRFP-VP26 foci at the periphery of the nucleus (Fig. 5 and 9; see also movies S1 to S3 in the supplemental material). This phenomenon was, however, not observed in wt HSV-1-infected cells stained with VP16- and VP26-specific antibodies, in which VP26, but not VP16, was consistently observed in the large foci at the nuclear periphery (Fig. 8). In contrast, the colocalization of VP26 and VP16 in the cytoplasm late in infection was readily observed in both rHSV-RYC- and wt HSV-1-infected cells (Fig. 5 and 8). There are two possible explanations for this discrepancy: first, the fusion of ECFP to VP16 may have altered some of its biological properties, leading to its recruitment into the large mRFP-VP26 foci at the nuclear periphery. Second, it is conceivable that VP16 and VP26 similarly colocalized in wt HSV-1-infected cells but that the VP16-specific antibody employed did not detect VP16 when present in the

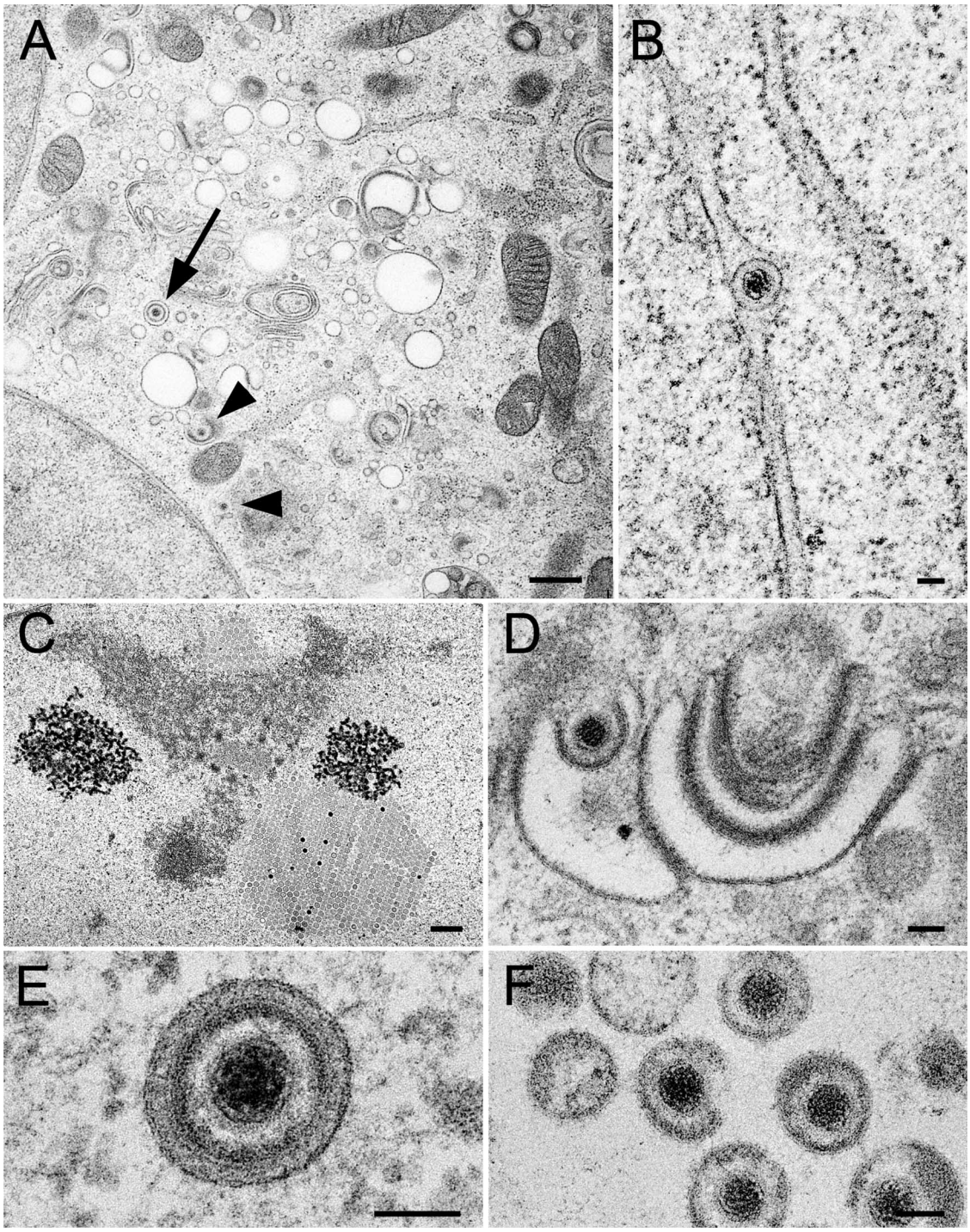

FIG. 10. Electron micrographs of Vero cells infected with rHSV-RYC after prefixation at 24 h p.i. followed by freezing and freeze-substitution. (A) Low-power micrograph showing Golgi membranes, virions within a vacuole (arrow), and capsids (arrowheads) within the cytoplasm and budding at Golgi membranes, respectively. (B) Virion within the perinuclear space of the nuclear envelope. (C) Accumulation of capsids in a crystalline manner within the nucleus. (D) Budding capsid at Golgi membranes. (E) Virion within a concentric vacuole. (F) Virions in the extracellular space. Bars, 500 nm (A and C) and 100 nm (B, D, and E).

large VP26 foci at the periphery of the nucleus, possibly because of an altered conformation of VP16 in these structures. However, the very late appearance of this colocalization (18 to 24 h p.i.) suggests that it likely does not represent a crucial step in the assembly and egress of progeny virus, since infectious progeny are produced already at earlier time points (i.e., 12 h p.i.) (Fig. 3). The accumulation of VP26 in large nuclear foci was previously documented in cells infected with wt HSV-1

and stained with VP26-specific antibodies, as well as by expression of VP26 fused to GFP. The formation of nuclear VP26 foci was demonstrated to depend on the presence of VP5 and VP22a and to occur in an ATP-dependent manner (8, 11, 13, 52). These VP26 foci were hypothesized to correspond to the sites of capsid assembly, similar to the assemblons described by Ward and coworkers, which contained the capsid proteins ICP35, VP5, and VP19c and formed very late in infection at the periphery of the nuclear RCs (13, 61). Interestingly, Ward et al. (61) detected a partial colocalization between assemblons and VP16 by staining with VP19c- and VP16-specific antibodies. However, this partial colocalization clearly differs from the almost perfect colocalization of VP16-ECFP and mRFP-VP26 in large foci at the nuclear periphery observed in our study (Fig. 5). The ultrastructural correlate of this colocalization remains to be determined. It is possible that the VP26 foci at the nuclear periphery may represent the accumulation of dead-end products, as previously suggested for assemblons (10, 36), to which VP16 is recruited late in infection. This hypothesis is further supported by the finding that these structures do not contain HSV-1 DNA, indicating that they do not correspond to accumulations of DNA-filled capsids (Fig. 7).

In summary, this study demonstrates the feasibility of the construction of a recombinant HSV-1 simultaneously expressing autofluorescent proteins fused to VP16, VP26, and gH. This study sheds light on the spatial and temporal organization of HSV-1 infection at the single-cell level. Specifically, the simultaneous fluorescence labeling of several virion components allowed assessment of the interactions of the different viral proteins in the course of the infection. This approach may be used to address several open questions in HSV-1 biology, for example, the spatial organization of capsid assembly and maturation by fluorescent labeling of several components of the pro-capsid and those of mature capsids. In addition, fluorescently labeled virus proteins may be combined with systems for the live visualization of viral DNA, as previously described for several viruses, such as HSV-1, Epstein-Barr virus, and adeno-associated virus (1, 23, 28, 29, 58), to specifically assess the dynamics of the association of viral proteins with viral DNA. Finally, the simultaneous fluorescent labeling of capsid, tegument, and envelope components may prove useful for the study of virus trafficking, for example, to assess and compare the composition of virions transported in an anterograde versus retrograde direction within axons (2–4, 20, 40).

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Herpes Simplex Virus Type 1/Adeno-Associated Virus Hybrid Vectors

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Abstract: Herpes simplex virus type 1 (HSV-1) amplicons can accommodate foreign DNA of any size up to 150 kbp and, therefore, allow extensive combinations of genetic elements. Genomic sequences as well as cDNA, large transcriptional regulatory sequences for cell type-specific expression, multiple transgenes, and genetic elements from other viruses to create hybrid vectors may be inserted in a modular fashion. Hybrid amplicons use genetic elements from HSV-1 that allow replication and packaging of the vector DNA into HSV-1 virions, and genetic elements from other viruses that either direct integration of transgene sequences into the host genome or allow episomal maintenance of the vector. Thus, the advantages of the HSV-1 amplicon system, including large transgene capacity, broad host range, strong nuclear localization, and availability of helper virus-free packaging systems are retained and combined with those of heterologous viral elements that confer genetic stability to the vector DNA. Adeno-associated virus (AAV) has the unique capability of integrating its genome into a specific site, designated AAVS1, on human chromosome 19. The AAV *rep* gene and the inverted terminal repeats (*ITRs*) that flank the AAV genome are sufficient for this process. HSV-1 amplicons have thus been designed that contain the *rep* gene and a transgene cassette flanked by AAV *ITR*s. These HSV/AAV hybrid vectors direct site-specific integration of transgene sequences into AAVS1 and support long-term transgene expression.

Keywords: HSV-1 amplicon vectors, adeno-associated virus, herpes simplex virus type 1, hybrid vectors.

HERPES SIMPLEX VIRUS TYPE 1 - BIOLOGICAL PROPERTIES

Herpes simplex virus type 1 (HSV-1) is a member of the family *Herpesviridae*, subfamily *Alphaherpesvirinae,* genus *Simplexvirus*. It is a common human pathogen that causes infections of the orofacial mucosal surfaces and may rarely cause acute hepatitis, kerato-conjunctivitis or meningo-encephalitis.

The HSV-1 particle is composed of three different compartments, capsid, tegument, and envelope. The capsid proteins are involved in the formation and maturation of the icosahedral capsid [1] and packaging of the viral genome [2-6]. The tegument, which is located between capsid and envelope, is composed of viral proteins involved in transport of capsids to nuclear pores, attachment to the nuclear pore complex [7], release of the virus genome from the capsid into the nucleus [8], and remodeling the host cell environment to optimize replication [9-14]. The viral envelope is a lipid bilayer of host origin that contains 11 viral glycoproteins. These play important roles in viral attachment, entry, cell to cell spread, and egress [15, 16]. HSV-1 can enter the cells by receptor-mediated fusion between virus and cell membrane [17-20]. However, depending on the cell type and virus strain, HSV-1 can penetrate the host cell also by endocytosis [20-22] and phagocytosis [23]. In the cytoplasm, the capsid is transported to the nucleus *via* interactions with the minus-end-directed microtubule motor protein dynein [24-26] Capsids bind to the nuclear pore complex and release the DNA genome into the nucleus [7, 27, 28].

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The HSV-1 genome is a double-stranded DNA (dsDNA) of 152 kbp. It is organized in two segments, unique long (UL) and unique short (US), both of which are flanked by inverted repeats (see Fig. **1**). The essential *cis* elements for viral DNA replication and encapsidation include the origins of DNA replication, located in the UL (ori_L) and TR_S, (ori_S) regions [29, 30], and the packaging/cleavage signals (pac) that reside in the *a* sequences located at both termini of the genome as well as at the junction between the long and the short segments [31]. The viral genome circularizes after it reaches the nucleus [32] and serves as a template for DNA replication. However, there is also evidence that circularization is not required for replication [33]. The majority of the replicative intermediates are long concatemers that are thought to have been synthesized by a rolling-circle mechanism [34-36]. The concatemers are cleaved into unit-length genomes at the *pac* signals after filling pre-formed capsids [36, 37].

HSV-1 encodes approximately 89 genes [38], which are expressed in a cascade of three temporal phases: immediate-early, early, and late. The late genes can be subdivided in leaky-late (expression is not dependent on viral DNA synthesis) and true-late (expression depends on viral DNA synthesis) [31, 39, 40].

There are several hypotheses on the mechanisms of envelopment of the nucleocapsid. The generally accepted view suggests a two-step envelopment process in which the capsid acquires a primary envelope by budding at the inner nuclear membrane and then is de-enveloped by fusion with the outer nuclear membrane [41, 42]. The secondary envelope is acquired when the capsid buds into the Golgi or cytoplasmic vesicles [43-49]. The alternative pathways described include (i) budding at the inner nuclear membrane followed by intraluminal transport *via* ER and Golgi and (ii)

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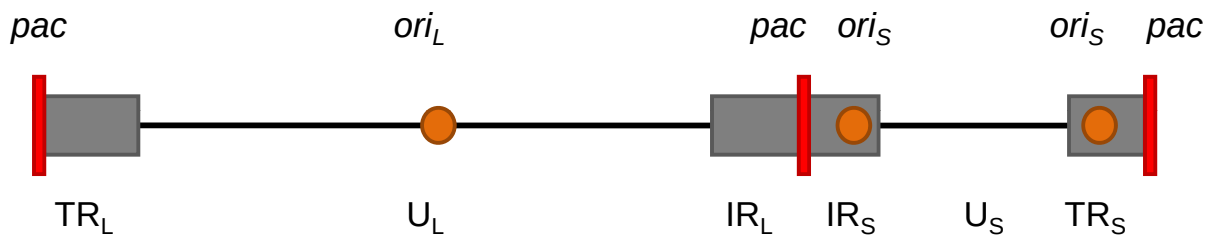

Fig. (1). Schematic representation of the HSV-1 genome. The HSV-1 genome is a linear double stranded DNA of approximately 152 kb in size, composed of two unique segments, U_L and U_S, which are flanked by inverted repeats, TR_L /IR_L and IR_S/TR_S. The minimal *cis* elements required for HSV-1 DNA replication and packaging include the origin of DNA replication, *ori,* and the cleavage/packaging, *pac,* signals.

exit *via* impaired nuclear pores and envelopment at the outer nuclear membrane or ER membrane [50-52]. Regardless of the mechanism of envelopment, mature virions seem to exit the cell by exocytosis *via* intraluminal transport to Golgi cisternae and formation of transport vacuoles [53-55].

An important aspect of HSV-1 biology is the capability of this virus to establish latent infections in sensory neurons of the trigeminal ganglia [56]. The latent HSV-1 genome is a circular, condensed episome, and viral gene expression is limited to the non-coding, latency-associated transcripts (LATs) [57]. Expression of LATs was demonstrated to increase the number of neurons in which latency is established [58] and to affect the efficiency of reactivation [59, 60]. Recent findings that LAT encodes several micro RNAs (miRNA) in HSV-1 infected cells corroborates with the proposed hypothesis that the exonic regions of LAT might function as primary miRNA precursors [61]. At least two of the identified miRNA precursors in latently infected neurons may facilitate the establishment and maintenance of viral latency by post-transcriptionally regulating viral gene expression [62-65].

Latent HSV-1 can periodically reactivate in response to a variety of stimuli, including fever, UV light, hormonal imbalance, malignancy or immune suppression, and enter a new lytic cycle, usually at the site of the primary infection. Recently, the requirement of ICP0 for viral DNA replication [66-68] and for exit from latency has been reconsidered, as *in vivo* studies showed that reactivation of HSV-1 genomes does not depend on viral DNA amplification [69] nor functional ICP0 [70]. Upon stress conditions, and in the absence of other viral proteins, VP16 was demonstrated to be activated [71], supporting the hypothesis that *de novo* expression of VP16 regulates entry into the lytic program in neurons. Repeated reactivation does not appear to kill the neurons, indicating that the extent of virus replication must be limited.

The understanding of the biological properties of HSV-1 and the molecular mechanisms of virus replication have allowed the design of specialized vector systems for somatic gene therapy, oncolytic virotherapy, and vaccination.

HSV-1 BASED VECTOR SYSTEMS

HSV-1 is an attractive vector for gene therapy due to its (i) large transgene capacity, (ii) high transduction efficiency and broad cell tropism that includes both dividing and non-dividing cells, and (iii) ability to establish latency while maintaining at least some transcriptional activity. However, as HSV-1 is a human pathogen, its use as a vector can result in host immune responses and cytopathogenic effects in patients, and possibly reactivation of and recombination with latent wild-type HSV-1. Taking these aspects into consideration, two different HSV-1-based vector systems, recombinant and amplicon, have been developed.

Initially, recombinant herpesviruses were constructed for functional studies of viral genes and development of vaccines. However, advances in site-specific modification of the viral genome facilitated the use of HSV-1 as a gene transfer vehicle [72]. Different approaches for the construction of recombinant HSV-1 vectors are based on the target tissue and purpose of gene delivery e.g. replication-conditional recombinant HSV-1 vectors are suitable for therapeutic treatment of tumors; replication-defective recombinant HSV-1 vectors are applied for gene replacement therapy [73]. Although some preclinical studies show promising results, several obstacles have to be overcome: (i) replication-defective mutants of HSV-1 can cause cytopathic effects in primary cultures of neuronal cells and inflammatory responses in neural tissue *in vivo*; (ii) most viral and nonviral promoters are silenced after injection into the brain. Therefore, the main focus in the development of new HSV-1-based vectors has been directed at achieving nontoxic, long-term gene expression in neurons.

The second type of HSV-1-based vector system, the HSV-1 amplicon vector, originated about three decades ago. Spaete and Frenkel analyzed the nature of defective virus genomes generated during the passage of standard HSV-1 stocks at high multiplicity of infection [74, 75]. Their investigations revealed that an *ori* and a *pac* signal were the only two *cis*-acting sequences required for the replication and packaging of defective virus genomes in the presence of *trans*-acting HSV-1 helpervirus (Fig. **2A**). The word amplicon was used to delineate the fact that multiple copies of a DNA sequence of interest can be amplified in a head-to-tail arrangement in concatemeric defective virus genomes and packaged into HSV virions [76]. HSV-1 amplicon vectors share similar structural and immunological properties with the wild type HSV-1 particle, which can trigger cell signaling and cellular responses that may have a transient impact on cell homeostasis or gene expression. However, the lack of virus genes and protein synthesis reduces the risk of reactivation, complementation and recombination with latent or resident HSV-1 genomes.

HSV-1 amplicon vectors have been used to infect efficiently a number of different cell types, including

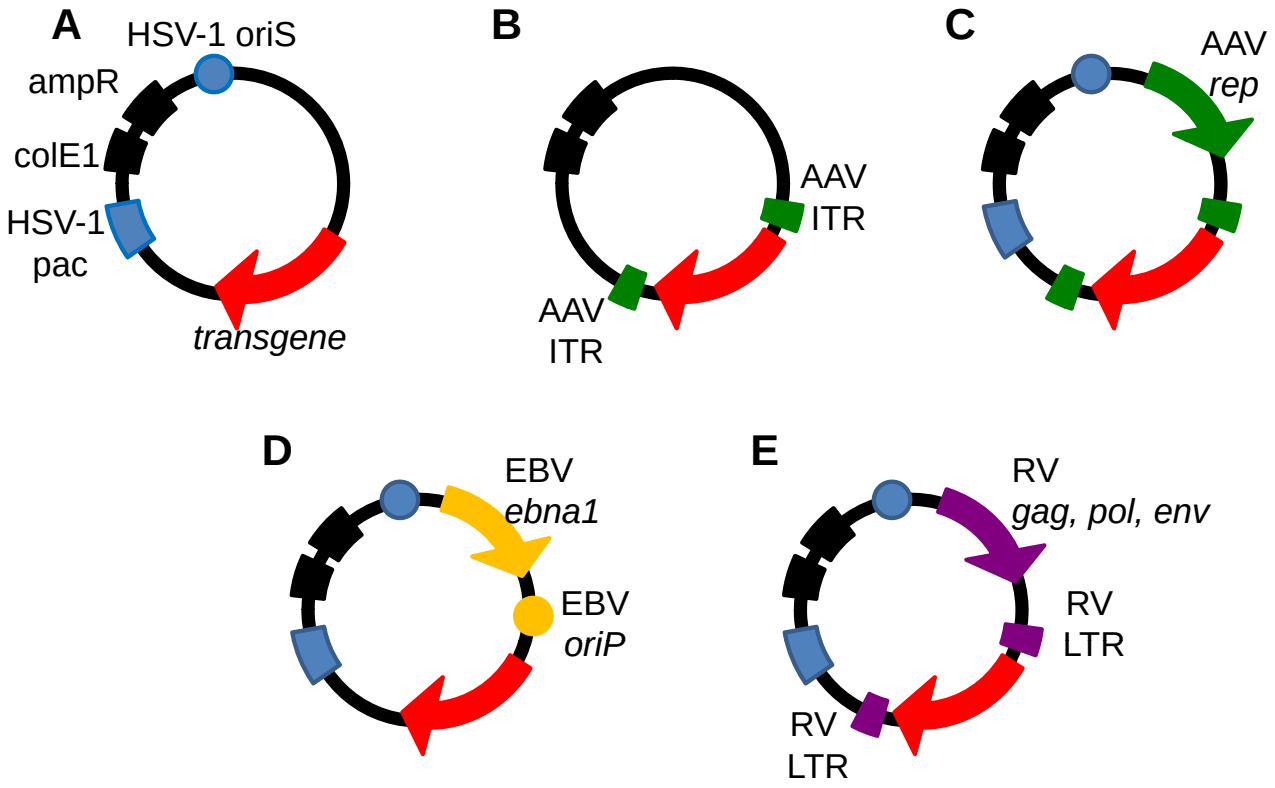

Fig. (2). Viral vectors. A) HSV-1 amplicon. The HSV-1 amplicon contains three types of genetic elements: i) sequences from bacteria (colE1 and ampR) that allow plasmid propagation in *E. coli*; ii) sequences from HSV-1, in particular an origin of DNA replication (*ori)* and a DNA packaging/cleavage signal (*pac*), which allow replication and packaging of the amplicon DNA into HSV-1 particles in the presence of HSV-1 helper functions in mammalian cells; and iii) a transgene cassette with the gene of interest. **B) Recombinant AAV vector.** Recombinant AAV vectors are bacterial plasmids that contain the AAV ITRs flanking a transgene of interest. Replication of the ITR cassette and packaging into AAV particles is achieved by supplying helpervirus functions and the *rep* and *cap* genes in *cis* or *trans* but outside the ITR cassette. **C) HSV/AAV hybrid amplicon.** In addition to the HSV-1 amplicon elements, HSV/AAV hybrid amplicon vectors contain the AAV *rep* gene and a transgene of interest flanked by AAV ITRs. **D) HSV/EBV hybrid amplicon.** In addition to the HSV-1 amplicon elements, HSV/EBV hybrid amplicon vectors contain the EBV origin of DNA replication (oriP) and the gene encoding EBNA-1 which together can support episomal retention and segregation of the vector in dividing cells. **E) HSV/RV hybrid amplicon.** In addition to the HSV-1 amplicon elements, HSV/RV hybrid amplicon vectors contain the retrovirus (MoMLV) *gag*, *pol*, and *env* genes, and the RV LTRs flanking a transgene of interest.

epidermal cells and dendritic cells in the skin [77, 78], some cell types in the cochlea [79, 80], hepatocytes [81], skeletal muscle [82], neurons [83], glioblastoma, and other tumor cells [84-86]. Despite the promising features of the HSV-1 amplicon vector as a gene delivery system, further developments concerning vector production, stability of transgene expression, and interaction with target cells are essential. Recently, the presence of bacterial sequences in the amplicon genome was shown to be responsible for the formation of inactive chromatin, leading to a rapid transgene silencing [87]. Strategies to increase the stability of transgene expression included the use of: (i) cell type-specific promoters [88, 89] and (ii) genetic elements from other viruses that confer genetic stability, such as integration of the transgene into host chromosomes [90, 91] or conversion of the amplicon genome into a replication-competent extrachromosomal element [92-95].

One of these strategies, the combination of genetic elements from HSV-1 amplicons and adeno-associated virus (AAV) to achieve site-specific integration of the transgene into the host genome and long-term transgene expression is described in detail below, after a short introduction into AAV biology.

ADENO-ASSOCIATED VIRUS – BIOLOGICAL PROPERTIES

Adeno-associated virus (AAV) belongs to the genus *Dependovirus* within the subfamily *Parvovirinae,* family *Parvoviridae* [96]. Different AAV serotypes have been identified that can infect a broad range of species; about 11 serotypes and more than 100 variants of AAV infect primates. Based on serological studies, AAV serotypes 2, 3, and 5 most probably have a human origin [97, 98], while AAV4 appears to have originated in monkeys [99]. AAV6 shares some genomic similarities with AAV2 and AAV1, raising the hypothesis that a recombination event could have occurred *in vivo* or in cell culture [100, 101]. The AAV serotypes 1 to 6 were isolated as contaminants in laboratory adenovirus stocks, while AAV 7, 8, 9, 10, and 11 were isolated as DNA molecules using a "signature PCR", a

screening-based strategy [102]. Despite being widespread among species and infecting different tissues, AAV infections have not been associated with any pathology. Primate AAV serotypes share significant sequence similarities, and the occurrence of cross-reaction of neutralizing antibodies may be species specific or depend on tissue type or route of administration [101, 103, 104].

The genome of AAV is a linear single stranded DNA of 4.7 kb, and either the positive or negative strand can be packaged with equal efficiency. The genome is flanked by inverted terminal repeats (*ITR*) of 145 bp, containing a palindromic sequence that forms a hairpin as a T-shaped secondary structure. The Rep binding site (RBS) and the terminal resolution site (TRS) are regions within the *ITR*s that play important roles in the replication and packaging of the AAV genome [105] (Fig. **3A**). Two open reading frames (ORFs), Rep and Cap, are responsible for encoding overlapping proteins through alternative splicing (Fig. **3B**). The Rep proteins, Rep78/68, and Rep52/40 are transcribed from two different promoters, p5 and p19, respectively, and are involved in DNA replication, transcription, and chromosomal integration. The p5 promoter contains a TATA box, a RBS, a TRS, the Yin Yang 1 (YY1) binding site, and a downstream sequence that can form a hairpin structure. The RBS is involved in Rep-mediated promoter regulation activity [106, 107], thus in the absence of helper functions small amounts of Rep are expressed that bind to the p5 promoter inhibiting transcription [108, 109]. The regulatory activity of Rep seems to be involved in the maintenance of a constant ratio of Rep and Cap proteins during infection in order to keep the balance between AAV genome replication and packaging. The Cap ORF encodes three overlapping proteins, VP1, VP2 and VP3, from a single promoter, p40. These structural proteins compose the AAV icosahedral capsid whose diameter ranges from 18 to 26 nm [110].

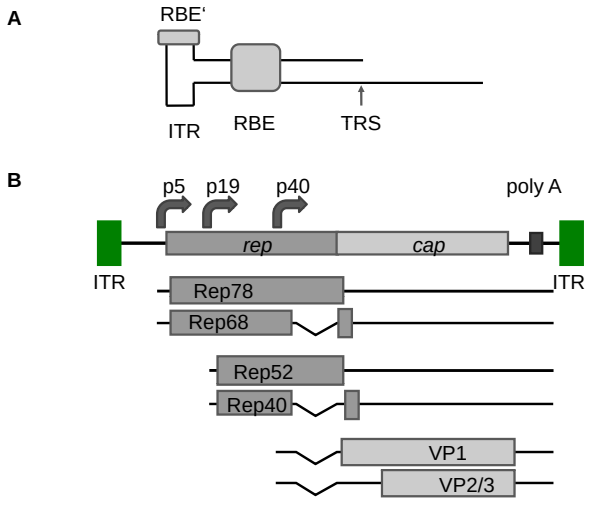

Fig. (3). Schematic map of the wild type AAV genome. A) Secondary structure formed by the inverted terminal repeat, ITR. Depicted are the Rep binding sites, RBEs, and the terminal resolution site, TRS. **B**) The AAV genome expresses two clusters of genes, *rep* and *cap*, from three different promoters, p5, p19, p40, by alternative splicing.

The replication of the AAV genome is based on a "rolling hairpin model". The hairpin structure at the *ITR* acts as a primer that converts the DNA into a double-stranded template, and together with the essential *cis*-acting elements RBS and TRS, and helpervirus functions, the replication and transcription of the AAV genome is initiated [111-113]. The Rep78/68 proteins play major roles in the replication process due to DNA-binding, endonuclease, and helicase activities. After binding to the RBS, Rep induces a site- and strand-specific nick at the TRS, creating a new genome end allowing the reinitiation of the synthesis and formation of a monomer extended form that can be packaged [114]. If the hairpin structure in the monomer turnaround form is not resolved before reinitiation on the other genome end, continued synthesis leads to double stranded dimer molecules, in which two genomes in the inverted orientations (head-to-head or tail-to-tail) are covalently joined by a single *ITR* [115]. Interestingly, the RBS and TRS signals located within the p5 promoter sequence have been demonstrated to act together as an alternative origin of DNA replication in the presence of adenovirus [116, 117] or HSV-1 helpervirus functions [108]. Replication from a plasmid cloned p5 replication origin led to the accumulation of large, head-to-tail linked concatameric replication products, which could readily be packaged into HSV-1 virions if the HSV-1 packaging/cleavage signal was included on the plasmid [108]. These findings indicate that the AAV p5 replication origin could substitute for the HSV-1 origin of DNA replication on HSV/AAV hybrid vectors (see below). AAV is a replication defective virus as it depends on a helper virus, such as adenoviruses [118], a herpesvirus [119-121], or vaccinia virus [122] for productive replication (Fig. **4**). Helper viruses are also responsible for inducing a cell cycle arrest in late S or G2 phase, as in the case of adenoviruses [123] or for down regulating host cell functions as in the case of HSV-1 as the helpervirus [124, 125].

Many studies have assessed the different elements from the helperviruses required for AAV replication. A model has been proposed where the HSV-1 helicase/primase proteins constitute a scaffold that recruits ICP8, Rep and cellular replication (RPA) proteins to the self-primed AAV DNA into replication compartments [126-129]. The HSV-1 polymerase complex is preferentially used for AAV replication instead of the cellular machinery [113, 130]. Interestingly, an inhibitory effect of Rep78/68 proteins has been described on HSV-1 replication [131, 132], suggesting a regulatory effect of AAV over HSV-1, thereby limiting expression of HSV-1 early genes [113, 129].

In the absence of helpervirus, the AAV genome can integrate into a specific site termed AAVS1, on chromosome 19q13.3-qter of human cells [133-136] (Fig. **4**). The integration is mediated by Rep78/68 and *ITR*s through a nonhomologous deletion/insertion recombination event [134, 136-143]. Also, an integration efficiency element (IEE) has been identified within the p5 promoter of AAV [144], and more specifically a 16-bp RBE was shown to be sufficient for AAV genome integration [145]. Rescue of the integrated AAV genome is possible by superinfection with helper virus [130]. Although HSV-1 ICP0 seems to contribute to the activation of the *rep* gene from latent AAV genomes [146],

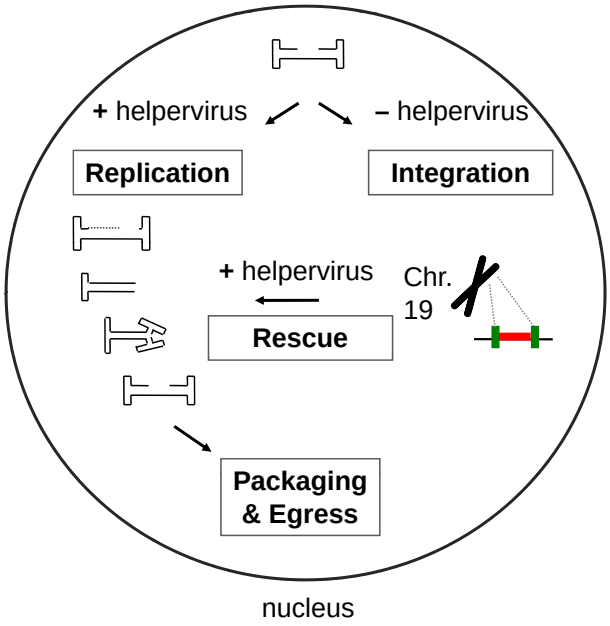

Fig. (4). The life cycle of AAV. Co-infection of AAV and helpervirus, adenovirus or HSV-1, leads to viral gene expression, viral DNA replication, and production of progeny virus. In the absence of helpervirus, the genome of AAV can integrate into a specific site on human chromosome 19. In the presence of helpervirus, integrated AAV genomes are rescued and enter the lytic replication cycle.

it is not sufficient to induce *rep* synthesis [130]. Some studies have demonstrated the autonomous replication of AAV under special conditions [147-149], however, the efficiency of replication is significantly lower than in presence of helpervirus functions.

AAV can infect different tissues and bind to unique cellular receptors, which can account for a serotype-specific tissue tropism. Several cellular receptors used by AAV for cell entry have been identified, including heparan sulfate [150], fibroblast growth factor receptor [151], and integrin alphaVbeta5 [152]. The initial steps of AAV infection, attachment to cellular glycosaminoglycan receptors and interactions with coreceptors seem to define the intracellular trafficking pathway of the capsid. Upon entry, AAV capsids are endocytosed *via* clathrin-coated pits [153], a process that requires dynamitin [154], and transported through both late and recycling endosomes. Trafficking in recycling endosomes appears to be favorable for efficient transgene expression [155]. The process of uncoating is still not well characterized [153, 156], however, AAV appears to enter the nucleus through a mechanism independent of the nuclear pore complex [157].

AAV BASED VECTORS

The broad cell tropism, lack of pathogenicity, and stable long-term gene expression make AAV an attractive vector for gene therapy [158]. AAV2 was the first AAV isolate to be developed into a recombinant vector for transgene delivery as it has been shown to infect a broad range of cell types in animal models [159], showing high efficiency in most of the tumor cells tested [160]. Recombinant AAV vectors are constructed by replacing the Rep and Cap ORFs with a transgene of interest flanked by the *ITR*s. Rep, Cap and helper functions can be supplied *in trans* in order to allow replication of the transgene in the host cell [135] (Fig. **2B**). Different methods of delivering helper functions have evolved, from co-infection with Ad or HSV-1 [161, 162], to plasmid-based protocols [163], and stable-expression by cell lines [164-166]. The development of a baculovirus based vector production method in insect (SF9) cells has also shown promising results [167, 168].

Recombinant AAV vectors have been tested in preclinical studies for a variety of diseases such as hemophilia, α-1 anti-trypsin deficiency, cystic fibrosis, Duchenne muscular dystrophy, rheumatoid arthritis, prostate and melanoma cancer, Canavan disease [169], Alzheimer's, and Parkinson's [170].

Recombinant AAV have shown efficient transduction of different regions of the brain, and are currently used in several clinical trials for neurological disorders [171-173]. Increased interest in designing AAV vectors for the treatment of neurodegenerative diseases that require gene delivery to broad areas or very local and specific areas of the brain are now the focus of many studies [174]. AAV2 has been the most widely used AAV serotype for gene delivery to the CNS, transducing almost exclusively neurons in different brain structures [175-178], and supporting long-term transgene expression in the CNS [179-181] as well as in the dorsal root ganglia [182]. AAV2 has shown higher transduction efficiency in glioblastoma *in vitro* and *in vivo* when compared to serotypes 4 and 5 [183]. However, other studies have demonstrated a higher distribution and transduction in the CNS when using rAAV serotypes 1 and 5 [175, 184, 185]. The different AAV serotypes have been exploited on their ability to efficiently transduce distinct regions of the brain due to different cellular tropisms [174, 183, 186].

Immune Response to rAAV Vectors

The brain has been thought to be an immune privileged, compartmentalized organ that lacks an adaptive immune response. Some studies have suggested that viral vectors induce little immunogenicity, especially when injected once in the parenchyma of naïve animals [104, 187, 188], or that the presence of antibodies to both capsid proteins and transgene products seems not to correlate with reduction in transgene expression [187, 188]. Further studies with pre-immunized animals, however, established that circulating neutralizing antibodies can affect intracerebral rAAV-mediated transduction, and even suggested that the adaptive arm of the immune system can be primed by intracerebral rAAV2 administration [104].

Immune responses in the absence of expression of AAV genes have also been observed in naïve animals in a dose-dependent manner [104]. This has been suggested to occur due to the slow process of AAV capsid uncoating, thus allowing antigen presentation of processed capsid peptides by MHC-I, or by an immune reaction specific to the transgene [189, 190].

Improvements on rAAV Vectors

Despite the explicit advantages of rAAV as a vector for gene therapy, improvements in the regulation of transgene expression need to be achieved in order to confer safety.

Much research is focused on efforts to limit vector spread, in order to achieve specific tissue or organ delivery, or to enable the transduction of tissues that are refractory to naturally occurring AAV vectors. Engineering of AAV vectors for altered tropism, enhanced transduction efficiency, and evasion of antibody neutralization includes manipulation of the AAV capsid by insertion of peptide ligands, conjugate-based targeting, and presentation of large protein ligands on the AAV capsid [191]. The diversity of AAV serotypes brings the possibility to evade preexisting immunity by engineering hybrid or pseudotyped AAV vectors derived from different serotypes [192-194].

Another strategy that focuses on the transduction efficiency is the improvement on the second-strand synthesis step during AAV replication (Fig. **5**). The development of self-complementary AAV (scAAV) vectors relies on the packaging of an inverted repeat genome that can fold into dsDNA without the requirement for DNA synthesis or base-pairing between multiple vector genomes, thereby bypassing the rate-limiting second-strand DNA synthesis [195]. The scAAV vectors displayed enhanced transduction in comparison with conventional AAV vectors in some tissues and cancer cells but their efficiency still depends on tissue, cell type, and route of administration [196, 197].

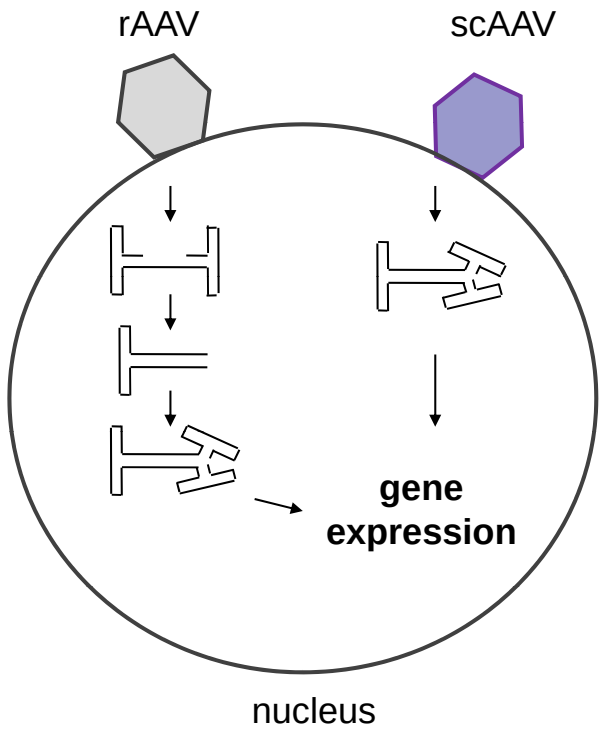

Fig. (5). Comparison between self-complementary AAV (scAAV) and rAAV vectors. scAAV delivers a dimeric inverted repeated DNA molecule thereby bypassing the rate-limiting second-strand DNA synthesis of rAAV.

Modifications on purification protocols using chromatography techniques have also contributed to increased yields of rAAV and to considerable elimination of contaminating infectious helper viruses [198-201].

HSV/AAV HYBRID VECTORS

Rationale on the Construction of Hybrid Vectors

Hybrid gene transfer vectors are designed to combine advantageous properties of different viruses to enhance efficiency of transgene delivery, vector stability and long-term transgene expression, while maintaining high safety standards [133, 202]. For example, the instability of HSV-1 amplicon vector delivered transgene DNA and transient transgene expression can be overcome by introducing genetic elements that allow the amplicon DNA to be maintained as an episome or to integrate into the host cell genome [203]. The maintenance of the DNA as a replicating episome with chromosome-segregating capability can be achieved by using oriP and the EBNA-1 gene from Epstein-Barr virus (EBV) [204]. Alternatively, viral elements such as AAV *ITR* and *rep* [91, 178], or retrovirus components [205] can be used to allow HSV-1 amplicon vector delivered transgenes to integrate into the cell genome. HSV-1 based hybrid vectors have also been constructed to facilitate the production of rAAV vectors.

Hybrid Vectors for the Production of rAAV Vectors

The efficiency of rAAV production for routine clinical use is a major concern, as most of the systems used for rAAV production rely on transfection protocols, thereby limiting scale-up procedures [206-209]. Replication defective rHSV-1 vectors lacking specific genes (e.g. ICP4, ICP27), which have been developed in order to reduce pathogenicity and cytotoxic effects in vector infected cells, can also be used as helper viruses for the production of rAAV vectors. Specifically, the ability of rHSV-1 that lack the ICP27 gene to efficiently act as a helper virus for rAAV production has been demonstrated [210] ; rAAV production in the absence of ICP27 appeared to be even enhanced. This may be due to the role of ICP27 in regulating transcription and translation of viral and cellular genes, for instance in the inhibition of splicing of host and AAV transcripts, which reduces synthesis of Rep and Cap proteins. The use of replication defective rHSV-1 to deliver AAV *rep* and cap has also been explored and is a very promising approach as it generates higher yields of rAAV with no detectable helpervirus contamination. Moreover, when allied to infection of a cell line that provides the rAAV template to be packaged, transfection steps can be avoided entirely for the production of rAAV [161, 211]. A protocol with a single infection step can also be accomplished by inserting an AAV *ITR*-flanked transgene (rAAV genome) cassette into the genome of the rHSV-1 helpervirus [210].

HSV/AAV Hybrid Vectors for Site-Specific Integration into AAVS1

Over the past 2 decades, the development of improved HSV-1 amplicon packaging systems, in particular the development of helper virus-free packaging systems, has greatly reduced toxicity and immunogenicity, but has had little effect on the stability of amplicon-mediated transgene expression [81, 212-214]. On the other hand, classical rAAV vectors have a small transgene capacity (~4.6 kb) and, due to the replacement of the *rep* and *cap* genes by transgenic sequences, do not conserve the potential of the parent virus for site-specific integration.

HSV/AAV hybrid amplicon vectors have been developed to overcome these limitations. In addition to the standard HSV-1 amplicon elements, HSV/AAV hybrid vectors incorporate the AAV *rep* gene and a transgene cassette that is flanked by AAV *ITRs* (Fig. **2C**). By placing the *rep* gene outside of the *ITR* cassette, it is not expected to integrate into the host genome. Loss of *rep* after integration of the *ITR* cassette eliminates a potential source of toxicity and the risk of rescue/excision of integrated *ITR* cassettes if the cell is infected by a helpervirus. Because HSV/AAV hybrid vectors can be packaged into HSV-1 virions, they conserve the high efficiency of gene transfer, the large transgene capacity, and the availability of helper virus-free packaging systems. However, after delivery into the host cell nucleus, the vector has the potential to act like AAV with *rep*-mediated site-specific integration of the *ITR*-flanked transgene cassette into the AAVS1 sequence of human chromosome 19 [91].

The initial study on HSV/AAV hybrid vectors demonstrated that these vectors can be packaged into HSV-1 virions by using either helper virus-dependent or helper virus-free packaging systems [81, 215]. Hybrid vectors supported transgene retention and expression significantly longer than standard amplicons [215]. Although the possibility of transgene integration had not been specifically addressed in that study, the percentage of cells expressing the transgene was consistently higher with hybrid vectors that contained the *rep* gene than with those without *rep*, or with standard amplicons.

Two other studies have specifically addressed the question whether HSV/AAV hybrid vectors mediate genomic integration, both randomly or site-specifically at the AAVS1 site on human chromosome 19 [91, 178]. Heister and colleagues constructed HSV/AAV hybrid vectors that contained enhanced green fluorescent protein (EGFP) reporter gene flanked by the AAV *ITRs* and AAV *rep*. Replication assays demonstrated that both the AAV elements and the HSV-1 elements were functional in the context of the hybrid vector, as shown by the presence of replication intermediates of the *ITR*-flanked transgene cassette and high molecular-weight concatemeric products of replication from the HSV-1 origin of DNA replication. Such hybrid vectors could be packaged into HSV-1 virions, although the *rep* sequences incurred a drastic (20 to 2,000-fold) reduction in titers. Site-specific integration at AAVS1 was directly demonstrated by PCR and sequence analysis of *ITR*-AAVS1 junctions in transduced human 293 cells. The junctions were similar to those that had been identified in cells infected with wt AAV [133, 134, 138, 139, 216-218]. Similar results were obtained by Wang and colleagues who have used also 293 cells and extended the study to other cell lines, including glioma cells (gli36) and primary myoblasts [178]. These investigators used HSV/AAV hybrid vectors that contained *rep68* and *rep78*, or no *rep*, and an *ITR*-flanked transgene cassette that consisted of an EGFP reporter gene and a neomycin resistance gene. In order to overcome the low-titer packaging problem inherent to the *rep* gene, they worked on position/orientation effects and found that a decent amplicon vector titer is achieved when the *rep* genes are placed downstream of the *ITR* cassette in the forward orientation. Rep mediated a significantly improved efficiency of stable tranduction in all human cells tested, including 293 cells, glioma cells and primary myoblasts. Although neomycin selection was employed for cell cloning, a high proportion of the stably transduced cells had the transgene sequences correctly integrated at the AAVS1 site. In summary, inserting the AAV *ITRs* and *rep* genes into an HSV-1 amplicon considerably improved the frequency of stable transgene expression in various proliferating human cell types. Integration events of 4-5 kb *ITR*-flanked transgene cassettes occurred at a rate of approximately 10-30 % of the HSV/AAV hybrid vector infected cells, and about 50% of those events occurred specifically at the AAVS1 locus [91, 178]. The potential for AAVS1-specific integration and expression of an entire gene under control of its endogenous promoter using the HSV/AAV vector has also been evaluated. Large functional inserts (approximately 100 kb) could be integrated at the AAVS1 site but with a reduced efficiency [219, 220].

While the expression of *rep78/68* has been demonstrated to be essential for the ability of HSV/AAV hybrid vectors to mediate site-specific integration, Rep proteins have a strong inhibitory effect on the HSV-1 replication machinery [91, 132, 178, 221]. As a consequence, the titers of HSV/AAV hybrid vectors are up to 2000-fold lower than those of standard amplicon vectors [91]. This could be due to (i) the toxicity of Rep, resulting in compromised cell metabolism [222], (ii) the ability of Rep to inhibit HSV-1 replication [131, 132], or (iii) the excision of *ITR*-flanked sequences from the amplicon DNA during packaging.

Potential improvements of the HSV/AAV amplicon vectors may rely on the appropriate use of the p5 promoter sequence. Indeed, the p5 promoter driving the expression of *rep78/68* in the afore described HSV/AAV hybrid vectors [91, 178] may promote vector-backbone integration [144, 223] owing to its location outside of the therapeutic transgene cassette. In addition, it may also interfere with site-specific integration of the p5-free *ITR*-flanked transgenes. Transferring the p5 promoter sequence from the *rep* expression cassette to the transgene cassette may not only solve the problem of inadvertent integration of vector backbone sequences but also increase the efficiency of site-specific integration of the *ITR* cassette [144, 223].

Liu *et al.* developed a strategy to overcome the negative effect of AAV Rep on hybrid vector replication and packaging [224]. These investigators designed an HSV/AAV hybrid vector in such a way that little or no *rep* was expressed during packaging. However, *rep* was expressed in transduced cells if Cre-recombinase was provided; following site-specific integration, *rep* was suppressed again. These vectors mediated stable expression in 22% of transduced Cre-expressing 293 cells. Of those cells, approximately 70% transduction efficiency was achieved by Rep-mediated site-specific integration.

The finding that concatameric plasmid replication products from the AAV p5 replication origin can be packaged into HSV-1 virions if HSV-1 *pac* is included on the plasmid [108] could lead to the construction of a novel generation of HSV/AAV hybrid amplicon vectors which replicate from a heterologous origin of DNA replication. Such a vector system would have several advantages: first, as described by Philpott and coworkers, the AAV p5 element can efficiently mediate site-specific vector integration into AAVS1 on human chromosome 19 and support long term

transgene expression [144, 225]. Second, the AAV p5 replication origin is not inhibited by *rep* expression, but instead depends on the presence of AAV Rep protein in the replication/packaging process [108, 116, 117].

The HSV-1 virion contains three proteinaceous compartments for delivery - envelope, tegument, and capsid – which could all be used to deliver functional foreign proteins by fusion with virion components [226]. For example, AAV Rep could be fused with VP16, an abundant HSV-1 tegument protein that enters the cell nucleus along with the virus genome. This would allow eliminating the *rep* gene from the HSV/AAV hybrid vector genome, as Rep protein could enter the cell nucleus as a fusion with VP16 and there may mediate efficient site-specific integration of the transgene sequences *via* p5 or *ITRs*.

The full potential of HSV/AAV hybrid vectors still needs to be evaluated for site-specific integration *in vivo*, for example in transgenic mice that carry the human-specific AAVS1 genomic element [227]. As murine [228] and simian [229] AAVS1 orthologs have been found, AAV2 likely can mediate site-specific integration in other species as well.

Future perspective and clinical use of HSV/AAV hybrid vectors are closely linked to standard HSV-1 amplicon vectors as both vector systems depend on the same packaging procedure. Helper virus-free packaging systems require transient transfection of vector DNA and packaging-defective HSV-1 helper DNA, which limits scale up potential. The use of amplicon vectors for clinical trials depends, therefore, on the design of novel packaging procedures that allow the production of large amounts of vector stocks with high titers. Strategies to overcome the adverse effects of the AAV rep gene on the titers of HSV/AAV hybrid vectors have been discussed above. The presence of the genetic elements from AAV on HSV-1 amplicon vectors should not add additional safety concerns to the amplicon system, as AAV is not known to be pathogenic in humans and AAV vectors are already being used in clinical trials.

OTHER HYBRID AMPLICON VECTORS

HSV/EBV Hybrid Vectors

Epstein-Barr virus, a human Gammaherpesvirus, has also been used as a hybrid partner with HSV-1 amplicons, due to its potential to persist as an extrachromosomal element in B-lymphocytes [230]. The EBV nuclear antigen (EBNA-1) and the origin of DNA replication (oriP) are the sole elements necessary for the long-term episomal retention and are therefore incorporated into the HSV-1 amplicon backbone to support replication and mitotic segregation of the amplicon concatenate in the host cell nucleus [231, 232] (Fig. **2D**). HSV/EBV hybrid amplicon vectors have been demonstrated to efficiently transduce various human cells in culture and to support retention of vector sequences in dividing human cells [205]. Stable expression from large transgenes has also been demonstrated [233, 234]. Maintenance of transgene DNA in an episomal state as opposed to genomic integration reduces adverse effects in the host cell. However, long-term expression by these vectors depends on selective pressure and expression of EBNA-1 [95, 235]. In order to circumvent the potential immunogenic and oncogenic properties of EBNA-1 [236], the use of a human episomal retention element (scaffold/matrix attachment region (S/MAR) from the human β-interferon gene to generate a novel HSV-1 amplicon-based episomal vector has shown great potential even in the absence of selection pressure [93].

HSV/RV Hybrid Vectors

Elements from retroviruses (RV) have been combined with HSV-1 amplicons in order to achieve prolonged transduction of transgenes. Retroviruses, such as Moloney murine leukaemia virus (MoMLV), integrate randomly into the genome of dividing cells, and produce viral progeny without killing the host cell [237]. Due to the low efficiency of gene transfer, MoMLV –based vectors have been mostly used for *ex vivo* gene therapy protocols [238, 239]. Although this strategy has shown some therapeutic success in experimental brain tumors [240, 241] it is not effective when used in human trials [242-245]. HSV/RV hybrid amplicon vectors containing genetic elements from MoMLV have been developed in order to transduce genes required for the *de novo* synthesis of small defective retrovirus vectors. These hybrid vectors contain the long terminal repeat sequences (LTRs) flanking a transgene cassette, and the *gag*, *pol*, and *env* genes in a separate cassette (Fig. **2E**). The LTRs and *psi* sequence comprise the signals necessary for packaging of virion RNA, reverse transcription, and integration into host cell genome. HSV/MoMLV hybrid vectors have indeed been demonstrated to support the packaging of genomic retrovirus RNA expressed from the amplicon vector into MoMLV particles and accomplish integration and transgenic expression in infected naïve cells [246]. One point of caution, however, is the danger of endogenous retroviruses complementing retroviral elements in hybrid vectors. The possibility that endogenous integrases can act on LTRs in hybrid amplicon vectors has indeed been demonstrated [247]. In order to enhance the transduction efficiency of a therapeutic gene *in vivo* and increase its expression stability, hybrid vectors containing elements from more than 2 viruses have been developed as well. These tribid vectors are based on HSV-1 amplicon vectors and contain elements from MoMLV and either EBV or AAV [205, 248].

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5. Publications

de Oliveira, A. P., and Fraefel, C. Herpes Simplex virus type 1/Adeno-Associated virus hybrid vectors. 2010. *The Open Virology Journal* 4 (14): 109-122.

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Silva, A. D., Sortica, V. A., Braga, A. C., Spilki, F. R., Franco, A. C., Esteves, P. A., Rijsewijk, F. A. M., Batista, H.B.C., de Oliveira, A.P., Roehe, P.M. 2005. Molecular and antigenic characterization of eight samples from Aujeszky disease virus isolated in the state of Rio Grande do Sul in 2003. *Pesquisa Veterinária Brasileira*, 25 (1): 21-24.

Spilki, F.R., Silva, A.D., Batista, H.B.C., de Oliveira, A.P., Winkelmann, E., Franco, A. C., Roehe, P.M. 2005. Field evaluation of safety during gestation and horizontal spread of a recombinant differential bovine herpesvirus 1 (BoHV-1) vaccine. *Pesquisa Veterinária Brasileira*, 25 (1): 54-58.

6. Proceedings

2nd Swiss Fundamental Virology Workshop. February 1st to February 2nd, 2010. Gerzensee, Bern, Switzerland. Visualization of HSV-1 DNA entry into the cell nucleus.Talk and Poster session.

33rd International Herpesvirus Workshop. July 27th to August 1st, 2008. Estoril, Portugal. Triple-fluorescent recombinant HSV-1 reveals dynamic events in the virus life cycle. Poster session 4.18.

32nd International Herpesvirus Workshop. July 7th to July 13th, 2007. Asheville, North Carolina, USA. Live visualization of HSV-1 compartment dynamics by multifluorescence confocal laser scanning microscopy. Talk and Poster session 4.18.

7. Courses/Workshops - Supervision

Mid-Term HEVAR (Herpesvirus based vaccines against rotavirus infections) Conference - Pratical Course of Virology. Co-supervision of a practical course dedicated to fundamental concepts of HSV-1 infection, production, titration and evaluation of amplicon vectors and recombinant vectors. Institute Pasteur - Montevideo, Uruguay. 7th to 14th April 2008 - 40 h

Master Student Semester Project Co-supervision. Co-supervision on semester project of a Master Student (Désirée Schönenberger) from ETH/University of Zurich, Zürich, Switzerland. September 15th to December 19th, 2008.